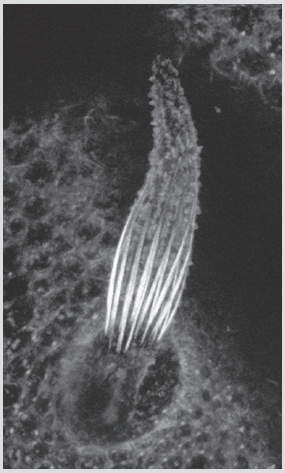

From Actin Monomers to Bundles: The Roles of Twinfilin and α-Actinin in *Drosophila melanogaster* Development

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Cover picture: Actin filament bundles stained with phalloidin
in a sprouting bristle from a *twinfilin* mutant pupa.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals, and on unpublished data presented in the text.

I **Wahlström, G., Vartiainen, M., Yamamoto, L., Mattila, P. K., Lappalainen, P. and Heino, T. I.** (2001). Twinfilin is required for actin-dependent developmental processes in *Drosophila*. *J. Cell Biol.* 155, 787-795.

II **Wahlström, G., Lahti, V. P., Pispa, J., Roos, C. and Heino, T.I.** (2004). *Drosophila* non-muscle α-actinin is localized in nurse cell actin bundles and ring canals, but is not required for fertility. *Mech. Dev.* 121, 1377-1391.

III **Wahlström, G., Norokorpi, H. L. and Heino, T. I.** (2006). *Drosophila* α-actinin in ovarian follicle cells is regulated by EGFR and Dpp signalling and required for cytoskeletal remodelling. Submitted manuscript.

ABBREVIATIONS

ABD	actin-binding domain
ACTN	α-actinin
ADF	actin depolymerising factor
ADF-H	ADF homology
ADP	adenosine diphosphate
A/P	anterior-posterior
Arp	actin-related protein
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CH	calponin homology
CNS	central nervous system
Dlar	*Drosophila* [homologue of] leukocyte common antigen-related
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
Dpp	Decapentaplegic
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	EGF receptor
ERK	extracellular signal-regulated protein kinase
F-actin	filamentous actin
FAK	focal adhesion kinase
FC-α-actinin	Follicle Cell-α-actinin
FLP	FLP recombinase
FRAP	fluorescence recovery after photobleaching
FRT	FLP recombination target
G-actin	globular actin
GdnHCl	guanidine hydrochloride
GFP	green fluorescent protein
GTPase	guanosine triphosphatase
hnRNP	heterogeneous nuclear ribonucleoprotein
Hts-F	Hu-li-tai-shao fusome
Hts-RC	Hu-li-tai-shao ring canal
kb	kilobasepairs
kDa	kilodalton
MAPK	mitogen-activated protein kinase
Mb	megabasepairs
MEK	MAPK kinase
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PtdIns	phosphatidylinositol

RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription PCR
Scar	suppressor of cAMP receptor mutation
UAS	upstream activation sequence
VASP	vasodilator-stimulated phosphoprotein
Wasp	Wiscott-Aldrich syndrome protein
Wave	WASP verprolin homologous protein

SUMMARY

The actin cytoskeleton is essential for a large variety of cell biological processes. Actin exists in either a monomeric or a filamentous form, and it is very important for many cellular functions that the local balance between these two actin populations is properly regulated. A large number of proteins participate in the regulation of actin dynamics in the cell, and twinfilin, one of the proteins examined in this thesis, belongs to this category. The second level of regulation involves proteins that crosslink or bundle actin filaments, thereby providing the cell with a certain shape. α-Actinin, the second protein studied, mainly acts as an actin crosslinking protein. Both proteins are conserved in organisms ranging from yeast to mammals. In this thesis, the roles of twinfilin and α-actinin in development were examined using *Drosophila melanogaster* as a model organism.

Twinfilin is an actin monomer binding protein that is structurally related to cofilin. In vitro, twinfilin reduces actin polymerisation by sequestering actin monomers. The *Drosophila twinfilin* (*twf*) gene was identified and found to encode a protein functionally similar to yeast and mammalian twinfilins. A strong hypomorphic *twf* mutation was identified, and flies homozygous for this allele were viable and fertile. The adult *twf* mutant flies displayed reduced viability, a rough eye phenotype and severely malformed bristles. The shape of the adult bristle is determined by the actin bundles that are regularly spaced around the perimeter of the developing pupal bristles. Examination of the *twf* pupal bristles revealed an increased level of filamentous actin, which in turn resulted in splitting and displacement of the actin bundles. The bristle defect was rescued by *twf* overexpression in developing bristles. The Twinfilin protein was localised at sites of actin filament assembly, where it was required to limit actin polymerisation. A genetic interaction between *twinfilin* and *twinstar* (the gene encoding Cofilin) was detected, consistent with the model predicting that both proteins act to limit the amount of filamentous actin.

α-Actinin has been implicated in several diverse cell biological processes. In *Drosophila*, the only function for α-actinin yet known is in the organisation of the muscle sarcomere. Muscle and non-muscle cells utilise different α-actinin isoforms, which in *Drosophila* are produced by alternative splicing of a single gene. In this work, novel α-*actinin* deletion alleles, including $Actn^{\Delta 233}$, were generated, which specifically disrupted the transcript encoding the non-muscle α-actinin isoform. Nevertheless, $Actn^{\Delta 233}$ homozygous mutant flies were viable and fertile with no obvious defects. By comparing α-actinin protein distribution in wild type and $Actn^{\Delta 233}$ mutant animals, it could be concluded that non-muscle α-actinin is the only isoform expressed in young embryos, in the embryonic central nervous system and in various actin-rich structures of the ovarian germline cells. In the $Actn^{\Delta 233}$ mutant, α-actinin was detected not only in muscle tissue, but also in embryonic epidermal cells and in certain follicle cell populations in the ovaries. The population of α-actinin protein present in non-muscle cells of the $Actn^{\Delta 233}$ mutant is referred to as FC-α-actinin (Follicle Cell).

The follicular epithelium in the *Drosophila* ovary is a well characterised model system for studies on patterning and morphogenesis. Therefore, α-actinin expression, regulation and function in this tissue were further analysed. Examination of the α-actinin localisation pattern revealed that the basal actin fibres of the main body follicle cells underwent an organised remodelling during the final stages of oogenesis. This involved the assembly of a transient adhesion site in the posterior of the cell, in which α-actinin and Enabled (Ena) accumulated. Follicle cells genetically manipulated to lack all α-actinin isoforms failed to remodel their cytoskeleton and translocate Ena to the posterior of the cell, while the actin fibres as such were not affected. Neither was epithelial morphogenesis disrupted. The reorganisation of the basal actin cytoskeleton was also disturbed following ectopic expression of Decapentaplegic (Dpp) or as a result of a heat shock.

At late oogenesis, the main body follicle cells express both non-muscle α-actinin and FC-α-actinin, while the dorsal anterior follicle cells express only non-muscle α-actinin. The dorsal anterior cells are patterned by the Dpp and Epidermal growth factor receptor (EGFR) signalling pathways, and they will ultimately secrete the dorsal appendages of the egg. Experiments involving ectopic activation of EGFR and Dpp signalling showed that FC-α-actinin is negatively regulated by combined EGFR and Dpp signalling. Ubiquitous overexpression of the adult muscle-specific α-actinin isoform induced the formation of aberrant actin bundles in migrating follicle cells that did not normally express FC-α-actinin, provided that the EGFR signalling pathway was activated in the cells.

Taken together, this work contributes new data to our knowledge of α-actinin function and regulation in *Drosophila*. The cytoskeletal remodelling shown to depend on α-actinin function provides the first evidence that α-actinin has a role in the organisation of the cytoskeleton in a non-muscle tissue. Furthermore, the cytoskeletal remodelling constitutes a previously undescribed morphogenetic event, which may provide us with a model system for in vivo studies on adhesion dynamics in *Drosophila*.

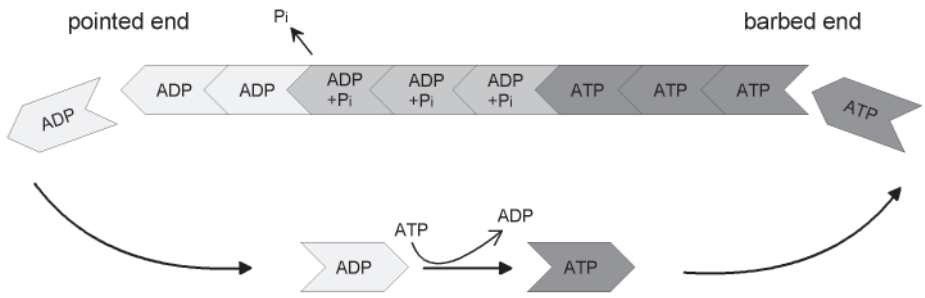

Figure 1. Actin treadmilling.

REVIEW OF THE LITERATURE

1. Introduction to the actin cytoskeleton

The actin cytoskeleton is one of three cytoskeletal systems that are present in eukaryotic cells, the other two being microtubules and intermediate filaments. Actin is the most conserved protein known among eukaryotes. Several actin isoforms with only subtle sequence variation exist within a species, but little is yet known about their distinct properties (dos Remedios et al., 2003; Röper et al., 2005). The actin cytoskeleton has been implicated in virtually all biological processes, ranging from muscle contraction to gene transcription (Clark et al., 2002; Pederson and Aebi, 2005). The actin cytoskeleton comes in many shapes, and depending on the context, it is either highly dynamic or structurally stable. Many of the functions attributed to the actin cytoskeleton are related to its ability to generate movement. Two very different mechanisms account for the generation of force: Extension of cellular processes is the result of actin filament polymerisation at the membrane (or at a particle in vitro), which physically pushes the membrane (particle) forwards (Pantaloni et al., 2001). The second mechanism relies on the movement of motor proteins (myosins) along actin filaments. In intracellular transport, cargo bound to myosin is transported along the actin filament, whereas actin and myosin filaments that slide along each other provide contractile force that can change the shape of the cell (Krendel and Mooseker, 2005). The actin cytoskeleton also has a structural role, in that stable actin filament bundles can provide the cell with a certain shape (Bartles, 2000). A large number of proteins regulate various aspects of the actin cytoskeleton, and they are in turn regulated by a number of mechanisms. Key mediators of the signalling pathways leading to cytoskeletal alterations are the Rho-family small GTPases Rho, Rac and Cdc42 (Bishop and Hall, 2000; Johndrow et al., 2004).

1.1. Actin treadmilling

Actin exists either as globular monomers (G-actin) or in a filamentous form (F-actin). Each actin monomer is bound to a nucleotide (ATP or ADP) and a divalent cation, usually Mg^{2+}. Actin filaments are polarised, with the opposite ends referred to as the barbed end and the pointed end. ATP-actin is preferably added to the barbed end of the actin filament. As the filament ages, ATP is hydrolysed to ADP, which promotes the release of ADP-actin from the pointed end. The ADP-bound actin monomer then undergoes nucleotide exchange, and the resulting ATP-actin is again ready to associate with another barbed end. This cycle of polymerisation, ATP hydrolysis and disassembly is called treadmilling (Fig.1), and it occurs spontaneously in the test tube under physiological conditions. However, in a living cell, the rate of treadmilling is more than one hundred-fold faster due to the large number of actin-binding proteins that catalyse each step of the cycle (dos Remedios et al., 2003).

1.2. Proteins regulating actin dynamics in vivo

The formation of a stable actin trimer is a very slow process, and it is therefore facilitated in vivo by actin nucleators. The actin nucleating activity of the Arp2/3 complex is attributed to the conformation of Arp2 and Arp3, which resembles that of an actin dimer. The Arp2/3 complex creates branched actin filament networks by binding to the side of pre-existing actin filaments and initiating growth of a new filament in a ~70° angle to the pre-existing one. Such actin filament networks are found within lamellipodia near the leading edge of migrating cells (Pollard and Beltzner, 2002). By contrast, formins and Spire nucleate the formation of long unbranched actin filaments. The formins remain bound to the barbed end of the actin filament where they function as leaky cappers, whereas Spire remains bound to the pointed end (Wallar and Alberts, 2003; Quinlan et al., 2005). Capping by tight cappers prevents both assembly and disassembly of actin filaments. Capping protein caps the barbed end of the actin filament (Wear and Cooper, 2004). Tropomodulin strongly binds and caps the pointed end in the presence of tropomyosin, a protein that stabilises actin filaments by binding to the side of the filament (dos Remedios et al., 2003; Winder and Ayscough, 2005). The actin filament severing protein gelsolin creates new capped barbed ends that can be elongated after uncapping (dos Remedios et al., 2003). ADF/cofilins (Actin Depolymerising Factor) increase the rate of actin depolymerisation at the pointed end. They also have a weak actin filament severing activity, and they inhibit spontaneous nucleotide exchange on monomers (Paavilainen et al., 2004). Profilin catalyses the nucleotide exchange of ADP for ATP on the monomer. In the presence of free barbed ends, profilin enhances actin polymerisation by delivering ATP-actin to the site of polymerisation, whereas in the absence of free barbed ends, profilin acts a monomer sequestering protein by preventing spontaneous nucleation (Paavilainen et al., 2004). Monomer sequestering activity is important in preventing spontaneous actin filament assembly in the presence of a high concentration of ATP-actin. In vertebrates, the β-thymosins are specifically dedicated to this activity (dos Remedios et al., 2003).

The above mentioned proteins (except for β-thymosin) have all been identified in *Drosophila* (Jacinto and Baum, 2003). For many of them, the phenotypes caused by mutations in the corresponding genes have been thoroughly characterised, while less is known about their biochemical properties in vitro. There are some examples of proteins that may have a different function in *Drosophila*, despite sequence homology to the corresponding yeast or mouse proteins. The amino acid sequence of *Drosophila* Profilin suggests that it may not catalyse nucleotide exchange, although mutational analyses did support a role for Profilin in promoting actin polymerisation (Benlali et al., 2000; Baum and Perrimon, 2001; Hopmann and Miller, 2003). Instead, a profilin-like activity was described for Ciboulot, a protein with sequence similarity to β-thymosin (Boquet et al., 2000).

1.3. F-actin structures

The architecture of F-actin structures is partly determined by the mode of actin nucleation and the efficiency of capping. This determines whether branched networks or long filaments will prevail (Mejillano et al., 2004). At this level, actin filament crosslinking and bundling proteins enter the scene to further regulate the three-dimensional architecture of the F-actin structure. Actin crosslinking and bundling proteins have at least two actin-binding sites, and the conformation of the protein determines whether the actin filaments will be crosslinked into networks, loose bundles or tight bundles. The filamin dimer contains multiple repeats that separate the dimerisation domain from the actin-binding site. Thus, filamin crosslinks actin filaments at a high angle (Stossel et al., 2001). By contrast, the globular protein fascin generates bundles of hexagonally packed unipolar actin filaments (Kureishy et al., 2002). A second bundle property that depends on the crosslinker is whether the crosslinked actin filaments will have the same or the opposite polarities (Winder and Ayscough, 2005). Unipolar bundles often have a structural role (Bartles, 2000), whereas filaments in bipolar bundles, as found in stress fibres and muscles, can slide along each other and generate force (Clark et al., 2002; Peterson et al., 2004).

2. Twinfilin

The actin monomer binding protein twinfilin was originally identified in yeast (*Saccharomyces cerevisiae*) through its sequence homology to ADF/cofilins (Goode et al., 1998). A mammalian homologue had been cloned already earlier and named A6, but it was erroneously classified as a tyrosine kinase (Beeler et al., 1994, 1997). Since then, three independent studies have failed to detect kinase activity in recombinant twinfilin (Goode et al., 1998; Rohwer et al., 1999; Vartiainen et al., 2000). Twinfilin homologues have been found in all eukaryotes except for plants (Palmgren et al., 2002). Twinfilin belongs to a large family of proteins that are characterised by the presence of an ADF homology (ADF-H) domain. Twinfilin is unique in having two ADF-H domains. ADF/cofilins have a single ADF-H domain, and they bind both G-actin and F-actin. Coactosin also has one ADF-H domain but binds only F-actin. Proteins in the drebrin/Abp1 class have an N-terminal ADF-H domain followed by additional sequences, and they also bind only F-actin (Lappalainen et al., 1998; Hellman et al., 2004).

2.1. Structure and in vitro activities

Twinfilin is a ~40 kDa protein that is composed of two ADF-H domains separated by a short linker and followed by a C-terminal tail. It binds monomeric actin in a 1:1 molar ratio with a ~10 fold higher affinity for ADP-actin than ATP-actin. In contrast to ADF/cofilins, twinfilin does not bind filamentous actin (Goode et al., 1998; Vartiainen et al., 2000; Palmgren et al., 2001; Ojala et al., 2002). The two ADF-H domains of twinfilin show only ~20% amino acid sequence identity to cofilin (Goode et al., 1998), but they are structurally similar. Analysis of the crystal structure of the N-terminal domain of mouse twinfilin revealed that twinfilin and cofilin bind ADP-actin using a similar interface, but that the regions in cofilin involved in F-actin binding are structurally different in twinfilin (Paavilainen et al., 2002).

In vitro, twinfilin acts as an actin monomer sequestering protein without affecting the rate of depolymerisation (Goode et al., 1998; Vartiainen et al., 2000). Twinfilin also inhibits nucleotide exchange on the actin monomer (Goode et al., 1998; Ojala et al., 2002). In yeast, both ADF-H domains are required for efficient actin monomer sequestering (Palmgren et al., 2001). In contrast, the C-terminal ADF-H domain of mouse twinfilin was as efficient as the full length protein in sequestering actin monomers. The C-terminal domain binds ADP-G-actin with a ~10 fold higher affinity than the N-terminal domain and has a dissociation rate constant almost 1/10 that of the N-terminal domain. The two domains also compete with each other and with cofilin for actin binding. This led to a model in which the ADP-actin monomer bound to cofilin is rapidly released and delivered to the N-terminal domain of twinfilin. A conformational change in twinfilin subsequently delivers ADP-actin to the C-terminal domain, which efficiently keeps it in its ADP-bound form (Ojala et al., 2002). Twinfilin's monomer sequestering activity is inhibited by PtdIns(4,5)-P_2 binding (Palmgren et al., 2001; Vartiainen et al., 2003).

2.2. Twinfilin in yeast

In yeast, twinfilin is localised in the cytoplasm and in the cortical actin patches but absent from the actin cables (Goode et al., 1998). Twinfilin's localisation to the cortical actin patches depends on its ability to bind both G-actin and capping protein. The interaction with capping protein takes place through the C-terminal tail of twinfilin. The proteins do not affect each other's actin-binding activities in vitro (Palmgren et al., 2001; Falck et al., 2004).

Yeast cells lacking twinfilin showed a brighter F-actin staining of the cortical actin patches and exhibited a random budding pattern. Viability was not affected, not even under stress conditions. In contrast, cells doubly mutant for the twinfilin deletion allele and a temperature-sensitive cofilin allele were larger than normal, exhibited severely reduced growth and a depolarised actin cytoskeleton together with enlarged cortical actin patches at the permissive temperature. The phenotype was suggested to be due to a synergistic depletion of the actin monomer pool (Goode et al., 1998). The phenotype of the double mutant cells could not be rescued by mutated twinfilin proteins deficient in either actin binding or capping protein binding, demonstrating that the interaction with capping protein is essential for twinfilin's role in actin dynamics (Falck et al., 2004). Overexpression of twinfilin resulted in a depolarisation of the cytoskeleton and the formation of aberrant non-filamentous actin structures (Goode et al., 1998).

2.3. Twinfilin in mammals

Mammals have two genes encoding twinfilin proteins that share ~65% sequence identity. Both twinfilins have similar actin-binding activities, bind to capping protein and are negatively regulated by PtdIns(4,5)-P_2. Twinfilin-1 is the more abundant isoform and is widely expressed. High levels of twinfilin-2 were detected only in heart tissue, and it is the only isoform present in skeletal muscle. The two isoforms showed a similar subcellular localisation pattern in mouse cell lines: punctated cytoplasmic staining and enrichment in those F-actin-rich areas that also contained G-actin. Overexpression of twinfilin-1 led to a decrease in stress fibres and an accumulation of abnormal F-actin structures. Different signalling pathways appear to regulate the subcellular localisation of the two twinfilins: Expression of an activated form of Cdc42 colocalised with twinfilin-1, but not twinfilin-2, at cell-cell contacts, and only twinfilin-1 accumulated in the membrane ruffles that were induced by activated Rac1 (Vartiainen et al., 2000, 2003). Mammalian twinfilins are also regulated by phosphorylation (Rohwer et al., 1999; Palmgren et al., 2002).

3. α-Actinin

The α-actinin protein was first isolated in 1965 and identified as a structural protein of striated muscle that could bind actin (Ebashi and Ebashi, 1965; Maruyama and Ebashi, 1965). In the muscle sarcomere, α-actinin is localised in the Z-disc, which serves as an attachment site for actin filaments of opposite polarities in adjacent sarcomeres (Clark et al., 2002). Ten years later, α-actinin was detected also in cultured non-muscle cells, where it was localised in a periodic pattern along the actin stress fibres as well as at cell-substrate and cell-cell attachment sites (Lazarides and Burridge, 1975).

3.1. The α-actinin gene family

α-Actinin belongs to the spectrin superfamily of proteins, which are characterized by having a varying number of spectrin repeats, an actin-binding domain and EF-hands. α-Actinin is considered an ancient member of the family (Broderick and Winder, 2005). α-Actinin is conserved among organisms ranging from fission yeast (*Schizosaccharomyces pombe*) and protozoa to humans. Baker's yeast (*Saccharomyces cerevisiae*) may have lost its α-actinin gene during evolution. The most primitive α-actinin-like proteins have only one or two spectrin-like repeats instead of four as in higher animals. It is thought that a primitive α-actinin appeared in the protozoan kingdom, and that intragenic duplication of the spectrin-repeats gave rise to modern α-actinin. Gene duplication in the vertebrate branch gave rise to four α-actinin genes (MacArthur and North, 2004; Virel and Backman, 2004). The different isoforms within a species show ~80% identity on the amino acid level, whereas the identity across different species (human and chick) for a particular isoform is over 90%. *Drosophila* α-actinin is ~70% identical to vertebrate α-actinins, and the slime mould *Dictyostelium discoideum* α-actinin is nearly 40% identical to both vertebrate and *Drosophila* α-actinin (Beggs et al., 1992; Imamura et al., 1994).

3.2. The structure of α-actinin

α-Actinin is a flexible rod-shaped molecule consisting of two antiparallel subunits with a molecular weight of approximately 100 kDa each (Winkler et al., 1997). Each monomer has three distinct domains: an N-terminal actin-binding domain (ABD), a central rod domain consisting of four spectrin-like repeats that mediate dimerisation (Djinović-Carugo et al., 1999; Ylänne et al., 2001) and a C-terminal calmodulin-like domain (Fig. 2). The ABD is composed of two calponin homology (CH) domains. Three regions within the ABD are important for actin binding, and they are followed by a phosphoinositide binding site (Gimona et al., 2002). The crystal structure of the ABD showed that the two CH-domains could adopt either a closed or an open conformation relative to one another, and it was suggested that this flexibility underlies the mechanism for regulation of actin binding by Ca^{2+} and phosphoinositides (Burridge and Feramisco, 1981; Fukami et al., 1992; Liu, J. et al., 2004; Franzot et al., 2005). A flexible linker separates the ABD from the central rod domain. The spectrin repeats have elastic properties and are able to react to mechanical stress by unfolding (Ortiz et al., 2005). The C-terminal calmodulin-like domain contains two EF-hand Ca^{2+}-binding motifs, each consisting of six amino acids

that coordinate a Ca^{2+} ion (Baron et al., 1987; Noegel et al., 1987). In the functional α-actinin dimer, the C-terminal domain of one monomer contacts the ABD of the other (Liu, J. et al., 2004; Franzot et al., 2005).

3.3. In vitro crosslinking and bundling

The flexibility of the α-actinin molecule allows for different modes of actin filament crosslinking. Direct visualisation of actin filaments crosslinked by α-actinin revealed that α-actinin can crosslink actin filaments into either networks or parallel bundles, and that the actin filaments within the bundle can have either the same or opposite polarities (Meyer and Aebi, 1990; Taylor et al., 2000). In addition, the angle between the α-actinin dimer and the actin filament is also highly variable (Taylor and Taylor, 1999). Bundle formation depends on the molar ratio of α-actinin to actin, with bundling occurring at high α-actinin concentrations (Meyer and Aebi, 1990; Wachsstock et al., 1993). Different α-actinin isoforms display different bundling properties under identical experimental conditions (Endo and Masaki, 1982; Meyer and Aebi, 1990).

α-Actinin's in vitro crosslinking activity has also been examined by measuring the viscosity of crosslinked F-actin gels and by analysing their elastic response to mechanical stress. Such experiments have shown that F-actin gels crosslinked by α-actinin are both stiff and elastic. They resist rapid mechanical stress, but in response to slow deformations, dynamic crosslinking allows for rearrangements within the F-actin network. α-Actinin crosslinks provide the F-actin network with a property termed strain hardening, which occurs when the force applied to the network results in increased elasticity. The factors that determine the mechanical properties of the crosslinked F-actin network are protein concentrations and the association-dissociation rate between α-actinin and F-actin (Sato et al., 1987; Xu et al., 1998, 2000; Tseng and Wirtz, 2001). In vitro, the latter can be regulated by temperature, whereas a corresponding in vivo regulatory mechanism may be provided by phosphoinositide binding to α-actinin (Fraley et al., 2005). F-actin networks in vivo contain several crosslinkers, which cooperate to produce an optimal combination of parallel bundles and networks. In vitro studies have shown that F-actin gels crosslinked by both α-actinin and fascin are stiffer and more elastic than gels containing only one crosslinker (Tseng et al., 2002a). By measuring the viscoelastic properties of the cytoskeleton in a living cell, it was demonstrated that injection of α-actinin altered the cell's response to mechanical stress in a manner similar to the response observed in vitro (Tseng et al., 2002b, 2005).

Figure 2. The α-actinin dimer. ABD = actin-binding domain; R1-R4 = spectrin-like repeats 1-4; EF = EF-hand domain.

3.4. α-Actinin isoforms in vertebrates

Vertebrates, or at least chicken and mammals, express several α-actinin isoforms encoded by separate genes. ACTN1 and ACTN4 encode non-muscle isoforms, whereas ACTN2 and ACTN3 encode muscle-specific isoforms (Virel and Backman, 2004). The only biochemically defined difference between them is that the non-muscle α-actinin isoforms have an EF-hand motif capable of binding Ca^{2+}, whereas the EF-hands are nonfunctional in the muscle-specific isoforms (Burridge and Feramisco, 1981; Arimura et al., 1988). Further diversity is created by alternative splicing. Alternative splicing within the EF-hand domain of ACTN1 generates a smooth muscle α-actinin isoform that does not bind Ca^{2+}, in contrast to the cytoskeletal Ca^{2+}-sensitive splice variant (Waites et al., 1992). The single chicken gene encoding skeletal muscle α-actinin is alternatively spliced in a similar way, producing two different Ca^{2+}-insensitive isoforms (Parr et al., 1992). In mammals, two sarcomeric isoforms are encoded by separate genes, ACTN2 and ACTN3. Of these, ACTN2 appears to be the homologue of chicken sarcomeric α-actinin. Expression of ACTN3 is restricted mainly to skeletal muscle, whereas ACTN2 is present in both skeletal and cardiac muscle (Beggs et al., 1992). ACTN2 is also expressed in other tissues besides muscles (Wyszynski et al., 1998; Mills et al., 2001; Kos et al., 2003). The most recently discovered member of the α-actinin gene family is ACTN4 (Imamura et al., 1994; Hsu et al., 1996; Honda et al., 1998). Both non-muscle α-actinin isoforms are co-expressed in several tissues, but they have distinct functions in vivo (Araki et al., 2000). Recently, a splice variant of ACTN4 was detected in certain lung cancers and in testis. The variant α-actinin-4 had a higher affinity for F-actin than the conventional α-actinin-4. The alternatively spliced exons encode part of the linker between the ABD and the first central repeat (Honda et al., 2004). This splicing pattern is also found in *Drosophila* and *Caenorhabditis elegans* *α-actinin* genes (Roulier et al., 1992; MacArthur and North, 2004).

3.5. Regulation of α-actinin function

3.5.1. Ca^{2+}-binding

Vertebrate non-muscle α-actinins, but not muscle-specific α-actinins, display a reduced F-actin binding activity and do not induce F-actin gelation in the presence of Ca^{2+} (Burridge and Feramisco, 1981; Duhaiman and Bamburg, 1984). However, although both α-actinin-1 and α-actinin-4 are regulated by Ca^{2+}, the two isoforms differ in their sensitivity. Chicken lung α-actinin, which corresponds to α-actinin-4, was characterised as "low-Ca^{2+}-sensitive type non-muscle α-actinin". Its gelation activity and binding ability towards F-actin was reduced in the presence of Ca^{2+}, but the difference was not as dramatic as seen with α-actinins isolated from other tissues, i.e. α-actinin-1 (Imamura and Masaki, 1992; Imamura et al., 1994). One of the two α-actinin isoforms isolated from human blood platelets and α-actinin isolated from rabbit macrophages were also reported to be less sensitive to Ca^{2+}-regulation (Landon et al., 1985; Pacaud and Harricane, 1993).

The biological significance of Ca^{2+}-mediated regulation of actin crosslinking has been investigated mainly in *Dictyostelium discoideum,* which expresses a Ca^{2+}-sensitive α-actinin (Condeelis and Vahey, 1982; Noegel et al., 1987). EF-hand I has a low affinity for Ca^{2+} and acts as a regulatory site for Ca^{2+}-inhibition, while EF-hand II has a high affinity for Ca^{2+}. When EF-hand II was mutated, higher than physiological Ca^{2+}-concentrations were required for inhibition of actin crosslinking, but the mutant protein was still able to rescue the phenotype of an α-actinin-gelation factor double mutant. Thus, it was suggested that Ca^{2+}-regulation of actin crosslinking might be dispensable in vivo (Witke et al., 1993).

Ca^{2+} may not only regulate binding of α-actinin to F-actin, but also interactions between α-actinin and other proteins (Chapters 3.6.3. and 3.6.4.).

3.5.2. Phosphorylation

α-Actinin-1 is phosphorylated on tyrosine 12 in the ABD by focal adhesion kinase (FAK), and this reduces α-actinin's affinity for actin filaments (Izaguirre et al., 2001). Dephosphorylation of α-actinin-1 is accomplished by the tyrosine phosphatases SHP-1 (Lin et al., 2004) and PTP 1B (Zhang et al., 2006). Furthermore, phosphorylation of α-actinin creates a binding site for the tyrosine kinase Src, and it was suggested that this initiates a feedback loop that regulates the phosphorylation status of FAK (Zhang et al., 2006). α-Actinin may also experience phosphorylation by serine/threonine kinases. An interaction between α-actinin-2 and the fatty acid- and Rho-activated serine/threonine protein kinase PKN was detected, and it was shown that PKN could phosphorylate α-actinin-2 in vitro (Mukai et al., 1997).

3.5.3. Phosphoinositide-binding

Vertebrate α-actinin interacts directly with several phospholipids, of which PtdIns(4,5)-P_2 and PtdIns(3,4,5)-P_3 have been shown to directly regulate the interaction between α-actinin and F-actin (Fukami et al., 1992; Greenwood et al., 2000; Fraley et al., 2003). In addition, PtdIns(3,4,5)-P_3, but not PtdIns(4,5)-P_2, disrupts the interaction between α-actinin and the integrin β subunit (Greenwood et al., 2000). The phosphoinositide binding site is located immediately on the C-terminal side of the third actin-binding site in the ABD, potentially also overlapping with it (Fukami et al., 1996; Gimona et al., 2002; Franzot et al., 2005).

The currently favoured view is that phosphoinositide binding inhibits α-actinin binding to F-actin. Fraley et al. (2003) found that both PtdIns(4,5)-P_2 and PtdIns(3,4,5)-P_3 inhibited the F-actin bundling activity of chicken smooth muscle α-actinin. Further characterisation of the inhibitory mechanism revealed that whereas both PtdIns(4,5)-P_2 and PtdIns(3,4,5)-P_3 prevented actin bundling by α-actinin, only PtdIns(3,4,5)-P_3 had the capability of disrupting F-actin bundles crosslinked by α-actinin. Since the two phosphoinositides induced different proteolysis cleavage patterns of α-actinin, it was suggested that they differently regulate α-actinin's flexibility (Corgan et al., 2004).

Expression of a mutated GFP-α-actinin with a reduced affinity for phosphoinositides induced the formation of abnormal actin bundles (Fraley et al., 2003), and FRAP microscopy revealed that the replacement of the mutant protein in bleached stress fibres and focal adhesions was slower than that of the wild type protein. Hence, it was suggested that phosphoinositides regulate the association-dissociation rate of α-actinin binding to F-actin and integrins (Fraley et al., 2005).

However, there are also studies reporting that PtdIns(4,5)-P_2 increases α-actinin's actin crosslinking activity. Fukami et al. (1992) found that α-actinin isolated from striated muscle contained large amounts of PtdIns(4,5)-P_2 and had a high F-actin-gelating activity. Smooth muscle α-actinin had a lower F-actin-gelating activity, but it increased following addition of exogenous PtdIns(4,5)-P_2. The gelation activity was slightly suppressed in the presence of a PtdIns(4,5)-P_2 phosphatase (Sakisaka et al., 1997). Analysis of the crystal structure of α-actinin-3 resulted in a model where PtdIns(4,5)-P_2 bound to the second CH-domain would interact with the linker sequence between the ABD and the first repeat, thereby displacing the C-terminal calmodulin-like domain from the ABD and allowing for actin binding (Franzot et al., 2005). A similar mechanism had earlier been suggested to activate α-actinin binding to the sarcomeric protein titin (Young and Gautel, 2000). It has been suggested that the apparent contradictions regarding the role of PtdIns(4,5)-P_2 may be explained by a two-step mechanism: Phosphoinositide binding to α-actinin would first cause a conformational modification in the ABD. Thereafter, hydrolysis of PtdIns(4,5)-P_2 into inositol 1,4,5-trisphosphate and diacylglycerol promotes binding to F-actin (Janmey and Lindberg, 2004). In agreement with this model is a report stating that diacylglycerol bound to α-actinin was absolutely necessary for actin bundling (Burn et al., 1985).

3.5.4. Regulation by proteolysis

Calpain 1, an intracellular Ca^{2+}-dependent protease, was found to associate with the C-terminus of both skeletal and smooth muscle α-actinin isolated from chicken. Nevertheless, only smooth muscle α-actinin was susceptible to cleavage (Raynaud et al., 2003). One of the two α-actinin isoforms in platelets is also cleaved by calpain (Gache et al., 1984). In vivo cleavage of α-actinin by calpain was found to correlate with a redistribution of α-actinin and the appearance of pseudopods in stimulated T-cells (Selliah et al., 1996).

α-Actinin proteolysis by urokinase gives rise to a molecule having quite an exotic function for an actin-binding protein. Blood cells grown on bone marrow extracellular matrix (ECM) was found to produce a monocyte/macrophage maturation promoting factor. The factor was identified as an N-terminal 31 kD fragment of α-actinin-4 and was named mactinin. Mactinin is found at sites of inflammation, where it attracts monocytes and promotes their maturation. Extracellular α-actinin, which has been deposited as footprint material in the ECM by migrating cells, is degraded to mactinin by the serine protease urokinase, which is secreted by the monocytes that enter the tissue during the inflammation response (Luikart et al., 1999, 2002, 2003; Masri et al., 1999).

3.5.5. Proteins affecting α-actinin activity

Some of α-actinin's binding partners have been shown to directly affect α-actinin's F-actin binding activity in vitro. Rabphilin, which is involved in Ca^{2+}-dependent exocytosis, was found to enhance the F-actin bundling activity of chicken smooth muscle α-actinin (Kato et al., 1996). Actinin-associated LIM protein (ALP) is a muscle-specific PDZ-LIM domain protein. ALP increases the F-actin bundling activity of α-actinin-2 by simultaneously binding to both the head and the rod domain, thereby stabilising the flexible linker between these two domains (Xia et al., 1997; Pashmforoush et al., 2001; Klaavuniemi et al., 2004). It was also suggested that ALP may restrict α-actinin's crosslinking orientation to the antiparallel conformation at the Z-disc (Klaavuniemi et al., 2004). Another member of the same family, Reversion-induced LIM protein (RIL), which is expressed in several non-muscle tissues, interacts with the extreme carboxy-terminus of α-actinin. This increased the binding of α-actinin to F-actin (Schulz et al., 2004; Vallenius et al., 2004).

3.6. Cell biological roles of α-actinin

3.6.1. α-Actinin in in vivo bundles

In the 70's and 80's, several studies were dedicated to describing the localisation of various cytoskeletal components in tissues. In such studies, α-actinin was detected in stress fibres of various cells in situ (Byers and Fujiwara, 1982; Wong et al., 1983; Drenckhahn and Wagner, 1986), in the cleavage furrow of dividing cells (Fujiwara et al., 1978) and in the circumferential bundle of the vertebrate intestinal brush border (Bretscher and Weber, 1978) and retinal pigmented epithelial cells (Drenckhahn and Wagner, 1985; Philp and Nachmias, 1985). These bundles are composed of antiparallel actin filaments and are able to contract in a manner similar to muscle fibres.

α-Actinin is localised along stress fibres in a periodic pattern. Peterson et al. (2004) analysed stress fibre dynamics using GFP-tagged α-actinin and regulatory myosin light chain. This analysis revealed that stress fibres differ from muscle sarcomeres in that α-actinin always occupies the part of the actin filament that is free of myosin filaments, and that α-actinin is recruited to or displaced from the stress fibre depending on whether the stress fibre stretches or contracts. This raises the possibility that the rate of stress fibre contraction could be regulated by modulation of α-actinin's concentration or affinity for F-actin (Janson et al., 1992).

Although α-actinin can create unipolar bundles in vitro, there are only a few examples of α-actinin being localised in a unipolar bundle in vivo. The comet tail of the intracellular pathogenic bacterium *Listeria monocytogenes* is a unipolar actin bundle that contains α-actinin (Dabiri et al., 1990; Temm-Grove et al., 1994). When *Listeria* movement was reconstituted in vitro, the tail appeared less dense in the absence of α-actinin, but the motility rate was not reduced (Loisel et al., 1999). In contrast, injection of the rod fragment of α-actinin into infected cells disrupted both the tail and bacterial movement

(Dold et al., 1994). The unipolar actin bundle within a filopodium is constructed via reorganisation of the lamellipodial F-actin network in which α-actinin is present. Nevertheless, only the proximal part of the filopodium contained α-actinin, while fascin was detected throughout the filopodium (Svitkina et al., 2003). Unipolar bundles of the microvillar type are typically crosslinked by two or three different actin bundling proteins, but so far, none of these have proven to be α-actinin (Bartles, 2000; DeRosier and Tilney, 2000).

3.6.2. α-Actinin as a regulator of cell migration

3.6.2.1. Mechanisms regulating cell migration

Cell migration is a complex process that requires coordination of a multitude of intracellular events. Most of the current knowledge of mechanisms that regulate cell migration comes from studies on cells growing on a flat surface. The steps in the migratory cycle are as follows: membrane protrusion driven by actin polymerisation at the leading edge, formation of cell-substrate adhesion sites near the leading edge, forward translocation of the cell body, disassembly of the adhesion sites near the rear end and finally retraction of the tail (Ridley et al., 2003). The rate of cell migration depends partly on the type of F-actin structure formed at the leading edge. Lamellipodial networks drive fast migration, whereas the formation of multiple long filopodia has an inhibitory effect on the migration rate (Krause et al., 2002). The second factor affecting the rate of cell migration is adhesion site dynamics. Both strong and weak adhesions correlate with low motility, whereas intermediate adhesion results in high motility (Ridley et al., 2003). For cells within a tissue, cellular motility also involves remodelling of cell-cell contacts (Gumbiner, 2005).

Both α-actinin-1 and α-actinin-4 have been implicated in the regulation of cell migration, although their different subcellular localisation patterns indicate that they play different roles in the process. Both α-actinin-1 and α-actinin-4 localise in stress fibres and lamellipodia, whereas only α-actinin-1 has been detected at cell-substrate adhesion sites. α-Actinin-4 is especially enriched in highly extended cell areas and in cell protrusions and has also been detected in the nucleus (Honda et al., 1998; Araki et al., 2000). At cell-cell adherens junctions, α-actinin-1 connects to cadherins via α-catenin (Knudsen et al., 1995; Nieset et al., 1997) and to nectins via Afadin DIL domain-interacting protein (ADIP) and LIM domain only 7 (LMO7) (Asada et al., 2003; Ooshio et al., 2004). One study addressing α-actinin's role in adherens junctions suggested that α-actinin is involved in cell separation (Guvakova et al., 2002). α-Actinin-4 appears to localise at cell-cell contacts only in certain cell types (Gonzalez et al., 2001).

3.6.2.2. α-Actinin-1 as a regulator of adhesion site dynamics

The mechanism whereby α-actinin-1 regulates cell migration mainly relates to its localisation at integrin-based cell-matrix adhesion sites, where it directly interacts with β-integrin (Otey et al., 1990). Integrins are transmembrane heterodimeric receptors that

are composed of an α-subunit and a β-subunit. Integrins bind to ligands in the ECM, and they interact with the actin cytoskeleton via linker proteins on the cytoplasmic side (Geiger et al., 2001). Aggregation of integrin receptors near the leading edge of a migrating cell induces the formation of small adhesion sites termed focal complexes. These will then either undergo turnover or develop into larger and more stable focal adhesions that extend stress fibres and translocate towards the centre of the cell. Finally, the adhesion sites disassemble at the rear end of the cell (Webb et al., 2002). Focal adhesions act as signalling centres that directly regulate cell behaviour, and more than 50 different proteins are currently known to be involved (Geiger et al., 2001; Wozniak et al., 2004). α-Actinin can bind directly to several adhesion site components (Otey and Carpen, 2004). The biological significance of some of these interactions has been verified experimentally (Table 1, and below).

α-Actinin is not present in the dynamic focal complexes, it is thus not required for adhesion as such. Instead, the recruitment of α-actinin to the adhesion site correlates with its stabilisation, growth and stress fibre extension (Edlund et al., 2001; Laukaitis et al., 2001; Zaidel-Bar et al., 2003; Peterson et al., 2004). It has been shown that with increasing α-actinin levels, the cells become more strongly adherent and less motile (Glück et al., 1993; Glück and Ben-Ze'ev, 1994). Calpain activity was shown to be necessary for α-actinin accumulation in the adhesion site. It was suggested that calpain cleavage promotes a conformational change within the adhesion site that allows for α-actinin binding to integrin (Dourdin et al., 2001; Bhatt et al., 2002).

The current view is that α-actinin is involved in adhesion site disassembly (Bhatt et al., 2002; Otey and Carpen, 2004), a process that is regulated via several different signalling

Table 1. Select binding partners for α-actinin in focal adhesions and their regulation by α-actinin

Binding partner	Proposed function for α-actinin	Method of study	Reference
Affixin/β-parvin	Interaction with α-actinin necessary for cell spreading.	Expression of an affixin/β-parvin fragment carrying the α-actinin binding site.	Yamaji et al., 2004
Vinculin	α-Actinin binding triggers a conformational change required for vinculin activation.	In vitro binding using an α-actinin fragment.	Bois et al., 2005
Zyxin	α-Actinin recruits zyxin to the adhesion site.	Expression of mutated zyxin; injection of a zyxin peptide into cells.	Drees et al., 1999 Reinhard et al., 1999 Li and Trueb, 2001 Nix et al., 2001

pathways and molecular mechanisms (Carragher and Frame, 2004). FAK is one of the key components regulating adhesion site turnover. One of the targets for phosphorylation by FAK is α-actinin. As long as α-actinin remains phosphorylated, it does not accumulate in the adhesion site (von Wichert et al., 2003). However, phosphorylated α-actinin together with FAK, Src and the tyrosine phosphatase PTP 1B have been suggested to be involved in a feedback loop that regulates the phosphorylation status of FAK, thereby contributing to adhesion site turnover and an enhanced rate of cell migration. Consistent with the model, a mutated α-actinin that could not be phosphorylated by FAK had no effect on the rate of cell migration (Zhang et al., 2006), even though it did localise in the adhesion site (von Wichert et al., 2003).

Another factor resulting in focal adhesion disassembly is loss of tension applied on the adhesion site by the connected stress fibre (Geiger et al., 2001). Since disruption of α-actinin located in an adhesion site was shown to disrupt the link between the adhesion site and the stress fibre (Rajfur et al., 2002), thereby reducing tension, a mechanism that removes α-actinin may directly contribute to adhesion site disassembly. Such a mechanism could be PtdIns(3,4,5)-P_3 binding to α-actinin, which was shown to disrupt the link between α-actinin and β-integrin (Greenwood et al., 2000). Treatment of cells with PtdIns(3,4,5)-P_3 or expression of constitutively active PI 3-kinase are two means whereby focal adhesion disassembly can be induced. When a mutated α-actinin with a reduced affinity for phosphoinositides was co-expressed with activated PI3K, the cytoskeletal reorganisation was partially inhibited. This observation thus provides further support for α-actinin's role in adhesion site disassembly (Greenwood et al., 2000; Fraley et al., 2005).

3.6.2.3. α-Actinin-4 in cell migration

Several studies have implicated α-actinin-4 as a regulator of cell motility and tumorigenicity. However, depending on the cell type, α-actinin-4 either enhances or reduces the motility rate. In human breast and colorectal cancer cells, α-actinin-4 expression has been correlated with high motility and tumorigenicity (Honda et al., 1998, 2005). In these cells, the mechanism might be related to the ability of α-actinin-4 to increase membrane ruffling. RNAi-mediated silencing of α-actinin-4 was shown to block the formation of ruffles and protrusions and to reduce cell motility. Conversely, activation of α-actinin-4 led to membrane ruffling and filopodia formation and increased motility (Lanzetti et al., 2004; Hayashida et al., 2005; Honda et al., 2005). In other cell types, the relation between α-actinin-4 expression and the motility rate was the reversed. Human neuroblastoma cells with high levels of α-actinin-4 did show membrane ruffling, but nevertheless, they were strongly adherent and had low tumorigenic potential. Cells with low α-actinin-4 levels were only weakly adherent and had a highly tumorigenic phenotype (Nikolopoulos et al., 2000; Menez et al., 2004). Furthermore, lymphocytes from ACTN4 knock-out mice displayed increased motility compared to wild type cells (Kos et al., 2003).

3.6.3. α-Actinin and vesicle trafficking

The actin cytoskeleton has been implicated in both endocytosis and exocytosis. α-Actinin has been found to accumulate under clustered surface molecules that subsequently undergo endocytosis (Geiger and Singer, 1979), to associate with several types of vesicles (Dubernard et al., 1997; Pol et al., 1997; Araki et al., 2000; Trifaró et al., 2002; Hegmans et al., 2004) and to directly interact with several proteins implicated in vesicle trafficking. One role for α-actinin in these interactions may simply be to connect the vesicle to the cytoskeleton, as was suggested for the interaction between α-actinin and rabphilin (Kato et al., 1996; Baldini et al., 2005). It has also been shown that internalisation and endocytosis of the Na^+/H^+ exchanger 3 (NHE3) are dependent on the formation of a protein complex that includes α-actinin-4, and it was suggested that Ca^{2+} binding to α-actinin-4 may induce the formation of this complex (Kim et al., 2002). In another study, RNAi-mediated depletion of α-actinin-4 disrupted the CART (cytoskeleton-associated recycling or transport) complex, which resulted in a significantly reduced rate of transferrin receptor recycling (Yan et al., 2005).

3.6.4. Regulation of protein activities by α-actinin

In addition to playing a role in cell migration, adhesion and vesicle trafficking discussed above, α-actinin has been implicated in a diverse array of other biological activities because of its direct or indirect association with various proteins. Table 2 lists those proteins whose activities could be modulated via manipulation of α-actinin.

The by far best characterised interaction is the one between α-actinin-2 and the *N*-methyl-D-aspartate (NMDA) glutamate receptors (Wyszynski et al., 1997). These receptors are ligand-gated ion channels located in the postsynaptic density of glutamatergic synapses in the brain, where they colocalise with α-actinin-2 (Wyszynski et al., 1998). The NMDA receptors have been shown to depend on the actin cytoskeleton for their function. According to a simplified model, α-actinin-2 links the NMDA-receptor to the cytoskeleton and contributes to the increased open probability of the channel in resting neurons. Receptor activation and Ca^{2+} influx allows Ca^{2+}/calmodulin to compete with α-actinin-2 for binding to the NMDA-receptor, thereby displacing α-actinin-2 from the receptor and causing receptor inactivation (Wyszynski et al., 1997; Zhang et al., 1998; Krupp et al., 1999; Rycroft and Gibb, 2004). α-Actinin's role in channel inactivation was investigated in cells transfected with the NMDA receptor subunits together with various α-actinin isoforms or constructs. Overexpression of full length Ca^{2+}-insensitive α-actinin isoforms or dominant negative constructs interfered with receptor inactivation, whereas a Ca^{2+}-sensitive isoform had no effect (Zhang et al., 1998; Krupp et al., 1999). This suggests that the inability of α-actinin-2 to respond to Ca^{2+} is an important property in the regulation of channel activity.

3.7. α-Actinin mutants

3.7.1. *Dictyostelium discoideum*

The only organism in which α-actinin has been extensively studied through mutagenesis is the unicellular slime mould *Dictyostelium discoideum*. Under certain conditions, the individual cells aggregate and differentiate into a spore-forming multicellular slug. Both life forms are motile. Complete lack of α-actinin had no negative effects on the cells, as long as they were grown or aggregated under standard laboratory conditions (Rivero et al., 1996, 1999; Fisher et al., 1997). However, α-actinin mutant cells did show altered cAMP signalling (Rivero et al., 1996) and exhibited a slight defect in adhesion on weakly adhesive surfaces (Weber, 1999; Ponte et al., 2000). Mutant cells exposed to lower temperature or increased osmolarity had a slightly reduced growth rate (Rivero et al., 1999), and they displayed a reduced elasticity in response to mechanical deformation (Eichinger et al., 1996). Specific developmental defects associated with the lack of α-actinin became evident only when the cells were plated and allowed to aggregate on soil plates, a substrate more similar to their natural habitat. It was suggested that an underlying defect in cell motility was the cause of the observed defects (Ponte et al., 2000).

Table 2. Proteins regulated by α-actinin

Protein	Direct interaction	Proposed function for α-actinin	Reference
Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)	Yes	Inhibits kinase activity by preventing Ca^{2+}/calmodulin binding to CaMKII.	Robison et al., 2005
G protein-coupled receptor kinases	?	Inhibits kinase activity.	Freeman et al., 2000
PI3K	Yes	Stimulates PtdIns(3,4,5)-P_3 production by colocalising the enzyme with its substrate PtdIns(4,5)-P_2.	Reséndiz et al., 2004
Cardiac L-type Ca^{2+} channel	?	Regulates channel activity.	Sadeghi et al., 2002
Voltage-gated Kv1.5 channel	Yes	Regulates channel activity.	Maruoka et al., 2000 Mason et al., 2002
Polycystin-2	Yes	Regulates channel activity.	Li et al., 2005
Glucocorticoid receptor interacting protein 1	Yes	Enhances gene transcription by acting as a coactivator for nuclear receptors.	Huang et al., 2004
DNaseY	Yes	Stimulates enzymatic activity and increases apoptosis.	Liu, Q. Y. et al., 2004

3.7.2. Human mutations

3.7.2.1. α-Actinin-4

The mammalian kidney appears to be the only organ absolutely dependent on α-actinin-4 function, as revealed by mutations in human and mouse ACTN4 (Kaplan et al., 2000; Kos et al., 2003). Nephrotic syndrome in general is a consequence of damage to the glomerular epithelial cells, or podocytes. Their role in the kidney is to maintain the filtration barrier between the capillaries and the proximal tubular lumen. The podocyte is a highly branched cell, and the distal so-called foot processes that extend into the tubular lumen are the main contributors to podocyte function. The foot processes are attached to the glomerular basement membrane via integrins and dystroglycans, and this area constitutes the filtration barrier. The filtration slits, which allow fluid passage between the interdigitating foot processes, are bridged by slit diaphragms. The slit diaphragm is a specialised type of cell-cell junction with similarities to both tight junctions and adherens junctions. The foot processes harbour a contractile actin cytoskeleton (Smoyer and Mundel, 1998; Oh et al., 2004). α-Actinin is localised in the prominent actin bundle within the foot process (Drenckhahn and Franke, 1988; Ichimura et al., 2003) and has also been found to interact with proteins that are components of the slit diaphragms (Patrie et al., 2002; Lehtonen et al., 2005).

A dominantly inherited form of focal segmental glomerulosclerosis (FSGS) with incomplete penetrance was shown to be caused by mutations in ACTN4. The identified mutations alter the sequence of the actin-binding sites or the linker region between the ABD and the central repeats. The mutated proteins all displayed increased co-sedimentation with actin filaments in vitro, and when expressed as GFP-fusion proteins in cultured podocytes, they appeared in large aggregates. It was suggested that podocyte malfunction results from toxic effects from the aggregates in combination with loss of normal α-actinin-4 (Kaplan et al., 2000; Yao et al., 2004; Weins et al., 2005). Mouse ACTN4 knock-out animals are born without obvious defects, although at a lower number than expected. The only observed abnormality in these mice was the development of FSGS by increasing age. The histological examination revealed an aberrant foot process morphology, in that the foot processes had retracted and were spread along the glomerular basement membrane. The slit diaphragms appeared normal (Kos et al., 2003). In contrast to the human kidney, mouse podocytes also express α-actinin-1 (Kaplan et al., 2000; Kos et al., 2003), but at least in cultured podocytes, its subcellular localisation differs from that of α-actinin-4 (Welsch et al., 2001).

3.7.2.2. α-Actinin-3

In human skeletal muscle, ACTN2 is expressed in all muscle fibres, while only a subset of the fibres express ACTN3. A nonsense mutation in the human ACTN3 gene resulting in a stop codon at position 577 is a common polymorphism within different ethnic groups. Approximately 18% of the world population is completely deficient in α-actinin-3 (North et al., 1999; Mills et al., 2001). This deficiency is not associated with

any defects and occurs also among top athletes. It has been suggested that the presence of α-actinin-3 is beneficial for rapid and strong muscle performance, whereas muscles lacking α-actinin-3 may have an increased ability to adapt in response to mechanical stress (MacArthur and North, 2004; Clarkson et al., 2005).

3.7.2.3. α*-Actinin-2*

A dominant mutation located near the N-terminus of α-actinin-2 was identified in a patient with a heart condition. The mutant α-actinin-2 no longer co-immunoprecipitated with its binding partner muscle-specific LIM protein (MLP), and it was unable to induce cytoskeletal changes when expressed in cells, as wild type α-actinin-2 did (Mohapatra et al., 2003).

3.8. *Drosophila* α-actinin

3.8.1. Gene structure

Drosophila sarcomeric and non-muscle α-actinins are encoded by a single gene (Fyrberg et al., 1990). Alternative splicing generates one non-muscle isoform and two muscle-specific isoforms. The linker region between the ABD and the first central repeat is encoded by a different exon in the non-muscle isoform compared to the two muscle variants. The use of different splice donor sites in the muscle-specific exon results in either a long or a short muscle-specific transcript (Roulier et al., 1992). Furthermore, two separate promoters are used for the non-muscle and the muscle-specific transcripts (Fyrberg et al., 1998). The longer muscle isoform (larval muscle-specific α-actinin) is expressed in larval muscles and in adult head and abdominal muscles, whereas the shorter isoform (adult muscle-specific α-actinin) is expressed in adult indirect flight muscles and tubular jump and leg muscles (Vigoreaux et al., 1991; Roulier et al., 1992). All three isoforms have identical C-terminal EF-hands, both of which according to the sequence were predicted to bind Ca^{2+} (Roulier et al., 1992). However, no Ca^{2+}-binding activity was detected in a blot overlay experiment (Dubreuil et al., 1991).

3.8.2. α*-Actinin mutants*

The *Drosophila* α*-actinin* (*Actn*) mutant alleles that have been examined to date include four point mutations that result in a flightless phenotype and several null alleles that are larval lethal (Perrimon et al., 1985; Homyk and Emerson, 1988; Fyrberg et al., 1990; Roulier et al., 1992). $Actn^{1}$ and $Actn^{2}$ have a single amino acid substitution within the first CH-domain and in the muscle-specific exon, respectively. These mutations give rise to normal or slightly increased levels of α-actinin, respectively. In $Actn^{3}$, the last nucleotide of the short muscle-specific exon is altered. This perturbs correct splicing, and consequently, only the longer larval muscle-specific isoform is produced. This isoform is apparently not fully stable in the flight and leg muscles, because the protein levels were much reduced compared to the wild type. In $Actn^{4}$, the splice acceptor site of

the last intron is mutated, and this severely reduces the amount of all α-actinin isoforms (Roulier et al., 1992).

The indirect flight muscles are most affected in *Actn*^{2} with discontinuous Z-discs and disrupted myofibrils, and least affected in *Actn*^{4}, in which only the peripheral myofibrils are not attached to the Z-disc. The terminal feltwork, which is the modified Z-disc of the terminal sarcomere that is connected to the tendon cell apposed to the cuticle, is more affected than the rest of the sarcomeres in all mutants. Heterozygotes for *Actn* null alleles also exhibited some defects in the terminal feltwork, although not severe enough to cause a flightless phenotype (Fyrberg et al., 1990). The myofibrils of newly eclosed *Actn*^{3} flies were fairly normal, apart from a punctated appearance of the Z-disc, whereas in old flies, the Z-discs were severely disrupted or in some cases degenerated. In contrast, the myofibrils of the *Actn*^{4} mutant did not degenerate over time. Since these mutants both express a reduced amount of α-actinin, the observed difference was suggested to be due to the misexpression of the larval muscle isoform in *Actn*^{3} indirect flight muscles. It was suggested that α-actinin has only a minor role in myofibril assembly, but that it is important for stabilising and anchoring the actin filaments within the sarcomere (Roulier et al., 1992).

Actn null mutant larvae derived from heterozygous mothers or from homozygous mutant germline cells die during the first or second day of larval life (Perrimon et al., 1985). Newly hatched larvae are flaccid and move poorly. They fail to grow and eventually become completely paralysed. As in the flight muscles, the myofibrils of the body wall muscles become progressively more disorganised, with the appearance of electron-dense rods and networks (Fyrberg et al., 1998). Defects in tissues other than muscles have not been reported. The lethality of *Actn* null mutants can be rescued by ubiquitous expression of the adult muscle-specific isoform, which thus is capable of substituting for the larval muscle isoform in larval body wall muscles. A chimeric α-actinin carrying an additional spectrin repeat in the rod domain was also fully functional, whereas substituting either the ABD or the EF-hand domain with the corresponding sequences from β-spectrin or α-spectrin, respectively, did not rescue the lethality (Dubreuil and Wang, 2000).

4. Bristles as a model system in actin biology

The bristles that extend from the cuticle of the adult fly adult serve as mechano- or chemosensory organs. The small mechanosensory bristles on the thorax, or microchaete, are arranged in rows with regular spacing. The large bristles, or macrochaete, are fewer in number and form a stereotypic pattern on the thorax and scutellum. Each bristle is composed of four cells: the bristle cell, the socket cell, a neuron and a sheath cell. The most prominent part of the bristle, or the bristle shaft, forms as a cytoplasmic extension from the bristle cell within the pupal epithelium. A mature bristle is a hollow chitinous structure that tapers towards the tip, with ridges and grooves running along the bristle shaft. The shape of the mature bristle is determined by the actin filament bundles that run along the length of the developing pupal bristle just beneath the plasma membrane. The cell protrudes between the bundles, and these protrusions account for the ridges seen on mature bristles (Fig. 3) (Lees and Waddington, 1942; Lees and Picken, 1945; Overton, 1967; Appel et al., 1993; Tilney et al., 1995).

4.1. Development of the bristle

The sprouting bristle can be first detected at 32 hours after puparium formation. Elongation of the bristle shaft, which proceeds for 16 hours, is driven by actin polymerisation at the bristle tip. Microtubules present in the bristle shaft are important for initiation but play only a minor role in elongation (Geng et al., 2000; Tilney et al., 2000a). By 60 hours of pupal development, the chitin layer has been deposited, and no traces of the actin bundles remain (Tilney et al., 1996a).

4.1.1. Bundle formation

Throughout bristle elongation, actin bundles are assembled at the bristle tip. Small pimples at the apical surface are sites where actin filaments extend into the cortex from electron-dense material attached to the plasma membrane. In the presence of jasplakinolide, a drug that stabilises actin filaments, these actin filaments form true microvilli. These core bundles contain 6-10 actin filaments that are held together by an as yet unidentified

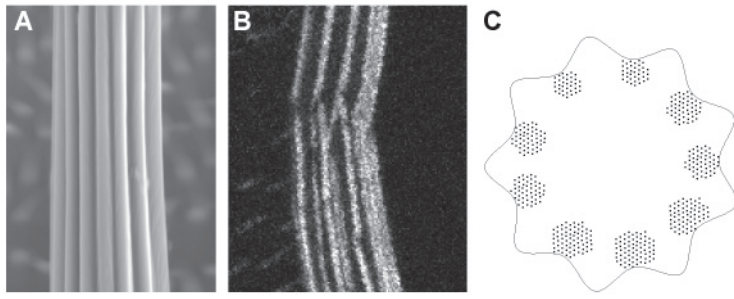

Figure 3. (A) Close-up view of an adult bristle. (B) Phalloidin-stained actin bundles in a pupal bristle. (C) Cross-section of a pupal bristle.

crosslinker (Tilney et al., 1996a, 2004). The next step involves aggregation of the core bundles into larger poorly organised bundles by the Forked protein (Petersen et al., 1994; Tilney et al., 1998, 2004; Wulfkuhle et al., 1998). Addition of Fascin, encoded by *singed* (Bryan et al., 1993; Cant et al., 1994), produces a maximally crosslinked bundle with hexagonally packed filaments (Tilney et al., 1995, 1998; Wulfkuhle et al., 1998). Further lateral addition of filaments increases the size of the bundles, which at the base of the bristle reach their maximum diameter between 35 and 38 hours of pupal development (Tilney et al., 1996a, 2000b).

The forming bundle modules, which are 1-2 μm in length, become aligned end-to-end separated by short gaps. Further actin polymerisation, which elongates the module to ca. 3 μm, fills in the gap and creates a small overlap between adjoining modules. So-called grafting of the modules, which involves addition of filaments, finally results in a smooth bundle that runs along the length of the bristle shaft without visible gaps. Nevertheless, the bundle is less stable at these gaps. Grafting depends mainly on the crosslinking activity of Forked, but to some extent also Fascin. Failed grafting is visible on the adult bristle as interruptions in the groove pattern or as short ridges and grooves running in any direction on the bristle shaft (Tilney et al., 1996a; Guild et al., 2003).

4.1.2. Bundles in the elongating bristle

Elongating microchaete have 7-11 bundles spaced around the perimeter of the bristle shaft, whereas in macrochaete the number is 16-25. The number of actin filaments per bundle is largest at the base, in microchaete up to 650 and in macrochaete up to 1000. All filaments have the same polarity, with the barbed end facing the tip (Tilney et al., 1995, 1996a, 1998, 2000b). The bundles are attached to the membrane along their sides. The molecule(s) mediating the attachment is not known, but the bundle-membrane connection was disrupted by treatment with a protein phosphatase inhibitor (Tilney et al., 2000b). Examination of various mutants with differently sized actin bundles revealed that the proportion of the membrane that contacts the bundles is constant, irrespective of the size of the bundle (Tilney et al., 2000b).

There is a continuous actin filament polymerisation and turnover going on in the elongating bristle. The snarls of actin filaments and tiny bundles that are present between the bundles and in the shaft cytoplasm have a very high turnover rate (Tilney et al., 2003). Incubation of developing bristles in jasplakinolide resulted in a significant increase in the number of filaments and small bundles in the bristle shaft. This treatment also increased the rate of bristle elongation, showing that mere polymerisation of actin filaments is sufficient to elongate the bristle. Blocking actin polymerisation with latrunculin A (sequesters monomers) or cytochalasin D (caps barbed ends and sequesters monomers) prevented bristle elongation (Tilney et al., 2000a, 2000b, 2003). The maximally crosslinked actin filaments in the membrane-attached bundles are less liable to turnover (Tilney et al., 2003). However, since stabilising filaments with jasplakinolide resulted in an elongation of the bundles into the cell body of the bristle cell (Tilney et al., 2003), also crosslinked actin filaments are presumably dynamic. FRAP experiments on bristles expressing

GFP-actin revealed a retrograde movement of the actin filaments within the bundles (Fei et al., 2002).

At around 40 hours of development, before the final length is reached (at 48 hours), the pointed ends of the filaments become capped. This conclusion was reached based on the observation that jasplakinolide-treatment of 41 hour old bristles no longer resulted in an elongation of the bundles into the cell body (Tilney et al., 2003). The punctated localisation pattern of the Capping protein β subunit in 44-50 hour bristles suggests that the barbed ends may also be capped (Hopmann et al., 1996).

4.1.3. Bundle disassembly

The actin bundle disassembly appears to be a reverse of the assembly process, except that the bundles remain maximally crosslinked throughout the disassembly process. The first sign of disassembly is that the overlaps between the bundle modules become visible. Then, by 43 hours of development, the overlaps develop into discrete gaps that enlarge as the breakdown proceeds. Live cell images of actin bundles decorated with GFP-moesin revealed that the enlargement of the gaps is accomplished by removal of actin subunits from the barbed end of each module. Module shortening was inhibited in the presence of jasplakinolide. Inhibition of protein synthesis with cycloheximide did not affect bristle elongation, but it induced premature bundle breakdown (Tilney et al., 1996a; Guild et al., 2002).

Bundle breakdown also involves longitudinal splitting of the bundle modules, which starts at 54 hours of development, and release of the subbundles into the cytoplasm. The membrane-attached part is the last to disassemble. Barbed end capping (by cytochalasin D), but not monomer sequestering (by latrunculin A), induced premature splitting into subbundles and separation into bundle modules. Gaps did not, however, form prematurely (Guild et al., 2002). By 60 hours of development, the actin bundles have disappeared (Tilney et al., 1996a).

4.2. Bristle mutants

How the actin crosslinking proteins Forked and Fascin contribute to bristle development has been extensively studied. It is also evident that a proper balance between actin polymerisation and turnover is critical for bundle formation, maintenance and disassembly. However, very little is known about the contribution of the individual regulators of actin dynamics to the various stages in bristle development. Several genes not directly implicated in actin regulation are also required for proper bristle formation (Table 3).

4.2.1. Crosslinking proteins

Mutations in *singed* or *forked* result in bristles that are shorter than normal and bent, branched or twisted with an irregular groove pattern. The explanation for the aberrant

Table 3. Genes required for normal bristle morphology (not in the text)

Gene	Protein product and/or function	Type of mutation	Phenotype	Reference
Stubble-stubbloid	Type II transmembrane serine protease	Dominant mutations / recessive mutations / heat shock-induced overexpression of protease-deleted constructs	Short thick bristles, increased number of actin bundles / frayed bristle ends / short bristles	Appel et al., 1993 Hammonds and Fristrom, 2005
skittles	Phosphatidylinositol 4-phosphate 5-kinase	Strong hypomorph or overexpression of UAS-*sktl*	Bent bristles	Hassan et al., 1998
rotund	RotundRacGAP (GTPase-activating protein)	Overexpression induced by a heat shock	Bent and split bristles	Guichard et al., 1997
Dral	Ral GTPase	UAS-GAL4-mediated expression of dominant negative Dral	F-actin accumulation in the bristle cell, but no outgrowth	Sawamoto et al., 1999
crinkled	Myosin VIIA	Loss-of-function	Deep ridges, projections on base of macrochaete, split and bent microchaete	Kiehart et al., 2004
mal-d	Cofactor for the transcription factor serum response factor (SRF)	Loss-of-function	Bent and split bristles	Somogyi and Rørth, 2004a
star	Processing of the EGFR ligand Spitz	Dominant gain-of-function mutation	Short and bent bristles	Ruden et al., 1999 Shilo, 2005
tricornered	Nuclear DBF2-related Ser/Thr protein kinase	Loss-of-function	Normal actin bundles, split bristles	Geng et al., 2000
furry	Component of the tricornered pathway	Strong hypomorph	Normal actin bundles, split bristles, bent head bristles	Cong et al., 2001
Datx2	Homologue of the spinocerebellar ataxia 2 (SCA2) gene product; putative regulator of RNA metabolism	Partial rescue of a null mutant by UAS-*Datx2* expression / *Datx2* overexpression	Split bristles, irregular groove pattern / ribbon-like bristles	Satterfield et al., 2002
small bristles	mRNA export protein	Strong hypomorph	Reduced diameter, loss of ridges	Korey et al., 2001
lark	RNA-binding protein	Mutated proteins expressed in a *lark* null mutant	Malformed scutellar bristles	McNeil et al., 2001

bristle formation in these mutants is that the actin bundles are not properly crosslinked and hence less stable (Tilney et al., 2003). The *forked* locus generates six different transcripts (Hoover et al., 1993), but the Forked D isoform alone is sufficient for normal bristle development (Grieshaber et al., 2001). In the *forked* mutant, a large number of tiny bundles form at the bristle tip, but most of them fail to aggregate and subsequently disappear. Consequently, the number of filaments per bundle is less than 1/10 the number seen in the wild type (Tilney et al., 1995, 1998). The bundles are disorganised and tend to lose their connection with the membrane (Grieshaber et al., 2001). Because of the Fascin crosslink, the filaments show hexagonal packing (Tilney et al., 1995). Since Forked is a limiting component in the bristle, an increased amount of Forked results in larger than wild type bundles that are irregularly shaped and hexagonally packed (Tilney et al., 2000b). In adult bristles, overexpression of *forked* is seen as branching of the bristle tip (Petersen et al., 1994). In the *singed* mutant, the early stages of bristle development appear normal (Wulfkuhle et al., 1998), but at later stages, irregularities in the bundle pattern become evident. The bundles contain only the Forked protein and will thus appear loosely packed. They also tend to line up against the membrane. The number of filaments per bundle is ~1/6 the number of filaments seen in the wild type (Tilney et al., 1995, 1998). Bristles from *singed-forked* double mutants contain a large number of short bundle modules in a variety of orientations (Wulfkuhle et al., 1998; Guild et al., 2003). In cross-sections, only single rows of actin filaments are seen attached to the membrane (Tilney et al., 1995). The fact that bundles form in the *singed-forked* double mutant shows that a third crosslinker in the bristle is yet to be identified (Tilney et al., 2004).

4.2.2. Proteins involved in actin dynamics

Hypomorphic alleles of the *chickadee* (*chic*) gene encoding Profilin (Verheyen and Cooley, 1994) and of *cpb* encoding the β subunit of Capping protein (Hopmann et al., 1996) both result in misshaped bristles. In the latter case, the groove pattern was also highly irregular. In both mutants, more abundant but thinner actin bundles than in the wild type were observed in the developing bristles. The F-actin level was increased in the *cpb* mutant bristles. In the *cpb* mutant, but not in *chic*, the bundles were often displaced from the membrane. Overexpression of Profilin resulted in a phenotype similar to the *cpb* mutant, and a reduction of the Profilin level in *cpb* mutant bristles suppressed the bristle phenotype. Thus, Capping protein and Profilin act antagonistically during bristle development: Capping protein limits F-actin assembly, while Profilin promotes actin polymerisation (Hopmann and Miller, 2003).

Drosophila Cofilin is encoded by the *twinstar* (*tsr*) gene (Gunsalus et al., 1995). A mutation that reduces the level of Cofilin was reported to result in a gnarled bristle phenotype. Developing bristles were not examined, but mutant cells in other tissues had elevated levels of filamentous actin (Chen et al., 2001). *Slingshot* (*ssh*) encodes a phosphatase that dephosphorylates Cofilin, thereby activating it. Loss of *ssh* function resulted in a higher level of phosphorylated (inactive) Cofilin in the cell, which in turn increased the level of F-actin. This resulted in split bristles with an irregular groove pattern (Niwa et al., 2002).

The Arp2/3 complex and its activator Wasp are involved in bristle development mainly by regulating the division pattern of the bristle precursor cell. Clones of cells in the eye disc lacking Arpc1 or Wasp (but not the second Arp2/3 complex activator Scar/Wave) resulted in bristle loss on the head (Zallen et al., 2002). Micro- and macrochaete were also lost in the absence of Wasp function (Ben-Yaacov et al., 2001). Once the bristle precursor cell is successfully established, Arp2/3 complex is not required for bristle elongation. However, mutant adult bristles possessed an increased number of longitudinal ridges (Hudson and Cooley, 2002a), which by inference means that the number of actin bundles was increased. The mechanism behind this phenotype has not yet been revealed. The involvement of the Arp2/3 complex in some aspect of bristle development was also demonstrated in a study on Sra-1 and Kette. These two proteins are part of a protein complex that represses Scar/Wave activity and thereby also the Arp2/3 complex. Reduced Sra-1 or Kette activity resulted in bent bristles, whereas expression of membrane-tethered forms induced F-actin accumulation in the bristle cell in a Wasp-dependent manner. This resulted in a split bristle phenotype on the adult cuticle (Bogdan and Klämbt, 2003; Bodgan et al., 2004).

5. Ovarian F-actin structures

5.1. Overview of oogenesis

The *Drosophila* oocyte is a ~0.5 mm large cell that contains all positional information required for setting up the anterior-posterior and dorsal-ventral axes of the early embryo. The oocyte is contained within an egg shell with several specialised structures (Fig. 4A). The oocyte develops within a structure called egg chamber (Fig. 4B). Each egg chamber contains one posteriorly located oocyte and fifteen highly polyploid nurse cells, all of germline origin. The role of the nurse cells is to provide the oocyte with all components required for early embryogenesis. The sixteen germline cells are surrounded by a single layer of somatic follicle cells. A chain of progressively older egg chambers connected to one another, and by the youngest egg chamber to the germarium, constitutes an ovariole, and 16-20 ovarioles together form one ovary (King, 1970).

Egg chamber assembly takes place in the germarium (Fig. 4C). The germline cyst arises from a single cystoblast, which undergoes four mitoses with incomplete cytokinesis. The cleavage pattern is directed by the fusome, which is a germline-specific organelle that extends through the arrested cleavage furrows and persists during these four divisions.

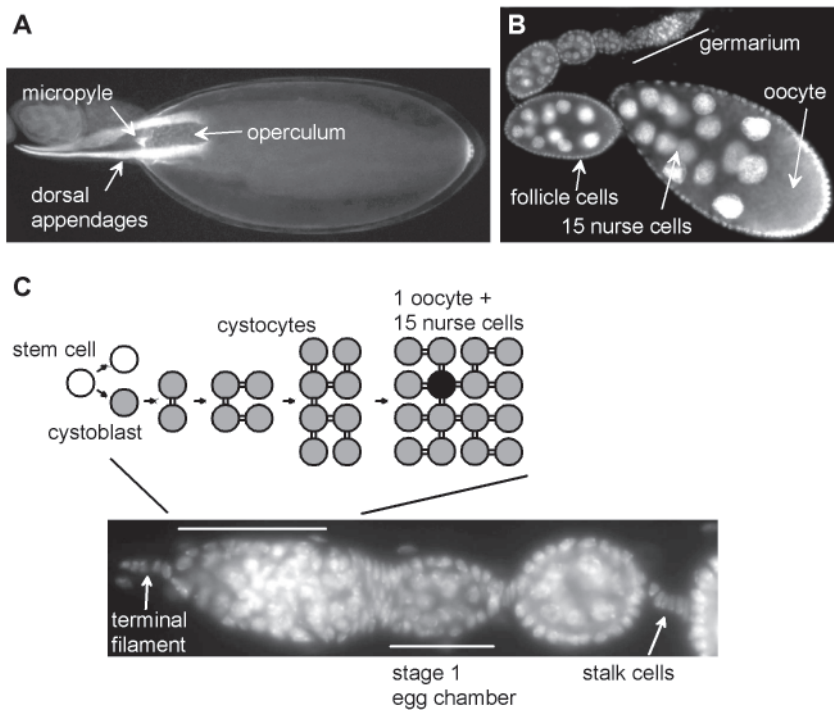

Figure 4. (A) A mature egg. (B) An ovariole stained with Hoechst showing the different cell types of the egg chamber. The largest egg chamber is a stage 9 egg chamber. (C) Egg chamber formation in the germarium.

The sixteen cells in the cluster remain connected to one another via actin-rich ring canals. One of the two oldest cells in the cluster differentiates into an oocyte, while the rest become nurse cells (Huynh and St Johnston, 2004). After completion of the mitotic divisions, the 16-cell cyst becomes encapsulated by follicle cell precursors that migrate from the germarium wall in between the germline cysts. A fraction of the follicle cells differentiate into stalk cells and polar cells, and this process results in the budding of a stage 1 egg chamber from the germarium (Horne-Badovinac and Bilder, 2005).

The subsequent development has been divided into 14 stages, where stage 14 refers to a mature egg. During stages 1-8, the egg chamber mainly grows in size and acquires an anterior-posterior (A/P) polarity. At stage 9, a majority of the follicle cells migrate posteriorly over the nurse cells to cover the oocyte (Horne-Badovinac and Bilder, 2005). A small group of cells, the border cells, detach from the anterior pole and migrate between the nurse cells until they reach the oocyte border (Montell, 2003). These cells will later contribute to the formation of the sperm entry site, or micropyle. At stage 11, the nurse cells rapidly transport all their cytoplasmic content into the oocyte in a process called dumping. Nurse cell dumping depends on proper organisation of three separate actin filament systems in the nurse cells: the ring canals, the cytoplasmic actin bundles and the contractile cortical actin cytoskeleton (Hudson and Cooley, 2002b). When dumping is completed, the nurse cell remnants are removed through apoptosis (McCall, 2004). Concomitantly with nurse cell dumping, the columnar follicle cells that cover the oocyte initiate a series of migratory events that creates the mature shape of the egg: The follicle cells at the anterior of the oocyte migrate between the nurse cells and the oocyte in a process called centripetal migration. These cells give rise to the operculum. Two groups of dorsolateral anterior follicle cells undergo a series of cell shape changes and a subsequent anterior migration. This results in the formation of two elongated tubes that will secrete the dorsal appendages (Berg, 2005). The rest of the follicular epithelium, referred to as main body follicle cells, flattens and expands in a process called egg elongation before secreting the chorion (Horne-Badovinac and Bilder, 2005).

5.2. Ring canals

The ring canals are actin-rich structures that assemble on the arrested cleavage furrows between the dividing cystocytes in the germarium. Throughout oogenesis, cytoplasmic components, and finally the bulk cytoplasm, are transported from the nurse cells through the ring canals into the oocyte.

5.2.1. Ring canal assembly

The ring canal is made up of two distinct layers: an outer rim of electron-dense material attached to the plasma membrane and an inner rim consisting of actin filament bundles and actin-associated proteins (Fig. 5) (Warn et al., 1985; Tilney et al., 1996b). The outer rim forms immediately following cytokinesis arrest during the four mitoses in region 1 of the germarium. Proteins known to accumulate on the arrested cleavage furrows are the microtubule-associated proteins Orbit/Mast (Máthé et al., 2003) and Pavarotti (Pav-

KLP) (Minestrini et al., 2002), the actin-bundling protein Anillin (Field and Alberts, 1995; de Cuevas and Spradling, 1998) and the glycoprotein Mucin-D (Kramerova and Kramerov, 1999). Antibodies against phosphotyrosine also label the outer rim (Robinson et al., 1994). In female sterile alleles of *orbit/mast*, fusome development is compromised, and Anillin and Pav-KLP fail to be recruited to the ring canals. This indicates that there is a connection between the fusome and the early stages of ring canal formation (Máthé et al., 2003). Anillin is encoded by *scraps*, in which several female sterile mutations have been isolated. However, since these females produce embryos (Field et al., 2005), the ring canals must have been functional.

As the cystocyte divisions are completed in region 2 of the germarium, F-actin (Warn et al., 1985), a product encoded by the *hu-li-tai-shao* gene (Hts-RC) (Robinson et al., 1994) and Filamin, encoded by *cheerio* (*cher*) (Li et al., 1999; Sokol and Cooley, 1999), are assembled into an inner rim. Filamin and Hts-RC are both required for initial F-actin accumulation, as the inner rim is missing in *hts* and *cher* mutants. This results in ring canal degeneration (Yue and Spradling, 1992; Robinson et al, 1997). Filamin recruitment to the ring canals does not require the presence of F-actin, since it localises to *hts* mutant ring canals (Sokol and Cooley, 1999). In contrast, Hts-RC does not localise to *cher* mutant ring canals lacking F-actin (Robinson et al., 1997). The mechanism by which Hts-RC tethers actin filaments to the ring canal is not yet known, whereas Filamin is thought to crosslink actin filaments both to one another and to the membrane (Li et al., 1999; Sokol and Cooley, 1999).

Addition of Kelch to the inner rim starts in region 3 of the germarium and is completed by stage 3 of oogenesis (Xue and Cooley, 1993; Robinson et al., 1994). This roughly coincides with the disappearance of Anillin by stage 2 (Field and Alberts, 1995). Kelch functions as a dimer that forms reversible crosslinks between actin filaments (Robinson and Cooley, 1997a; Kelso et al., 2002). The *kelch* gene encodes two protein products from a single transcript by stop codon suppression (Xue and Cooley, 1993). Although both products associate with the ring canals, the shorter product is sufficient for Kelch function in vivo (Robinson and Cooley, 1997b). In the absence of Kelch, the ring canal actin bundles become disorganised and obstruct the lumen of the canal (Robinson et al., 1994; Tilney et al., 1996b). A similar phenotype is seen in *importin-α2* (Gorjánácz et al., 2002) and *orbit/mast* (Máthé et al., 2003) mutants, which both fail to accumulate Kelch on the ring canals.

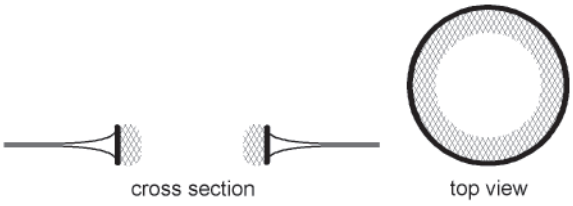

Figure 5. Cross section and top view of a ring canal. The outer rim is depicted as a thick black line. The hatched area corresponds to the actin-rich inner rim.

5.2.2. Ring canal growth

A newly formed ring canal is less than 1 μm in diameter, and by stage 11, the maximum diameter of 10 μm is reached. The actin filaments within the inner rim are loosely packed and of mixed polarities. Until stage 5, the number of actin filaments in the inner rim increases concomitantly with a slow growth of the ring diameter. It is not known how actin filament assembly is regulated during this early phase of ring canal growth. From stage 5 onwards, the diameter of the ring continues to increase, while the thickness of the inner rim remains constant. During this phase, it is thought that ring canal expansion takes place via polymerisation of new actin filaments and a concomitant sliding of the existing bundles within the ring canal (Tilney et al., 1996b). It has been speculated that ring canal expansion could involve the minus-end directed Myosin VI motor (Hudson and Cooley, 2002b). The transition stage between the two modes of ring canal growth coincides with the appearance of ring canal defects in egg chambers lacking Kelch (Tilney et al., 1996b) or either one of the Arp2/3 complex components Arpc1 or Arp3. Despite localisation of at least one of the Arp2/3 complex components already in the germarium, initial ring canal assembly and growth are normal in *Arpc1* and *Arp3* mutants. At later stages, ring canal collapse is observed, indicating that late ring canal expansion involves an Arp2/3 complex-mediated actin nucleation mechanism (Hudson and Cooley, 2002a). Arp2/3 complex function in the ring canals is mediated by Scar (Zallen et al., 2002).

Src64 and Tec29 tyrosine kinases are both required for early as well as late growth of the ring canal. F-actin, Hts-RC and Kelch localise properly in *Src64* and *Tec29* mutants, but tyrosine phosphorylation of ring canal proteins is reduced. Localisation of Tec29 to the ring canal depends on phosphorylation by Src64, Tec29 activity itself and a PtdIns(3,4,5)-P_3-mediated mechanism (Dodson et al., 1998; Guarnieri et al., 1998; Roulier et al., 1998; Lu et al., 2004). The defective ring canal growth in *Src64* and *Tec29* mutant egg chambers is largely due to failed reorganisation of the actin filament crosslinks by Kelch. The actin-binding activity of Kelch is inhibited by phosphorylation in a Src64-dependent manner. Expression of Kelch protein mutated in its phosphorylation site in a *kelch* mutant background phenocopied the *Src64* mutant phenotype (Kelso et al., 2002), which on the ultrastructural level appeared as a failure to separate the inner rim actin filaments into discrete bundles at stage 9 (Tilney et al., 1996b; Kelso et al., 2002). In the absence of Kelch phosphorylation, an increased amount of F-actin, which was less dynamic than in the wild type, accumulated in the ring canal (Kelso et al., 2002). Src activity may also be linked to Arp2/3 complex function via Cortactin, which is a Src substrate and a stimulator of the Arp2/3 complex, since *cortactin* mutants displayed a small ring canal phenotype (Somogyi and Rørth, 2004b).

Mutations that perturb the function of Myosin II also result in ring canal defects, although it is not yet clear how. There are conflicting reports regarding the presence of Myosin II in the ring canal (Edwards and Kiehart, 1996; Jordan and Karess, 1997). A fraction of the ring canals in germline cells lacking the regulatory myosin light chain encoded by *spaghetti squash* (*sqh*) appeared wrinkled (Jordan and Karess, 1997), and the ring canals in germline cells lacking myosin phosphatase activity failed to grow (Tan et al., 2003).

5.3. Nurse cell cytoplasmic actin bundles

The nurse cell actin bundles belong to the microvillar type of unipolar actin bundles (Bartles, 2000; DeRosier and Tilney, 2000). Their role is to anchor the nurse cell nuclei in the centre of the cell during nurse cell dumping at stage 11 (Fig. 6). If bundles fail to form, the nuclei will physically block the ring canals during dumping, resulting in a dumpless phenotype, i.e. short eggs.

5.3.1. Bundle structure

The first sign of actin bundle formation is at stage 6-7, when a basket consisting of tiny actin bundles forms around the ring canal. It has been proposed that these bundles might play a role in the slow cytoplasmic transport from the nurse cells to the oocyte during stages 7 to 10A (Riparbelli and Callaini, 1995). During stage 10B, the nurse cell bundles gradually assemble starting from the membrane, in greatest number in the vicinity of the ring canals. The bundles appear striated, because they are made up of several short subbundles that associate laterally with short overlaps. Each bundle contains ~25 hexagonally packed actin filaments that all have the same polarity. The barbed end is attached to the membrane in an electron dense patch, while the pointed end terminates a short distance away from the nuclear envelope. During nurse cell contraction, the bundles slide past one another, resulting in shorter and thicker bundles that no longer appear striated (Riparbelli and Callaini, 1995; Guild et al., 1997). These shortened bundles surround the degenerating nurse cell nuclei at stage 12, whereas at stage 13 they also traverse the nuclei (Gutzeit, 1986; Nezis et al., 2000). Incubation of egg chambers in cytochalasin D disrupted the subcortical actin filaments and prevented nurse cell contraction. In contrast, the nurse cell actin bundles remained unaffected (Gutzeit, 1986; Cooley et al., 1992), indicating that the actin filaments in the nurse cell bundles are not dynamic.

5.3.2. Actin-binding proteins in bundle formation

The two actin bundling proteins that crosslink the actin filaments of the nurse cell actin bundles are Quail and Fascin. Quail is a member of the gelsolin/villin family of actin-binding proteins. It contains six villin-like repeats and a carboxy-terminal headpiece (Mahajan-Miklos and Cooley, 1994). The headpiece is responsible for Quail's actin bundling activity (Cant et al., 1998). In contrast to vertebrate villin, Quail does not nucleate, sever nor cap actin filaments, and it is not regulated by Ca^{2+} (Matova et al., 1999). Quail is localised in the subcortical actin cytoskeleton of the nurse cells and the oocyte prior to stage 10, and from stage 10B, it localises to the nurse cell actin bundles. In mutants lacking

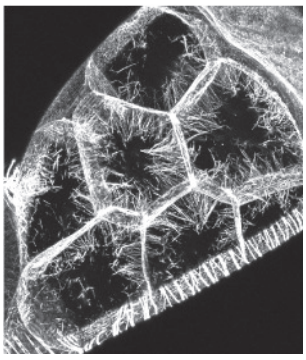

Figure 6. Nurse cell actin bundles in a stage 10B egg chamber.

Quail, the nurse cell subcortical actin layer is normal, but the nurse cell bundles fail to form (Mahajan-Miklos and Cooley, 1994). Egg chambers from *singed* null mutants also appear normal until stage 10B, when only some sparse and thin actin bundles form (Cant et al., 1994). Fascin has not been detected in the bundles, although the 12 nm period and the hexagonal packing of the bundles are indicative of Fascin crosslinking (Cant et al., 1994; Cant and Cooley, 1996). Overexpression of *quail* in a *singed* null mutant rescued bundle formation and fertility, demonstrating partial redundancy. However, the bundles were less rigid than in the wild type and did not fully support the nurse cell nuclei during dumping (Cant et al., 1998). Overexpression of *singed* did not rescue the *quail* mutant phenotype (Hudson and Cooley, 2002b). The only protein involved in actin dynamics that has been implicated in nurse cell actin bundle formation is Profilin, which is encoded by *chickadee* (*chick*). The *chick* gene produces two transcripts with identical open reading frames that are differently expressed. In female sterile *chick* mutants, the nurse cell-specific transcript is missing. During nurse cell dumping, the subcortical actin cytoskeleton contracts, but the nurse cell actin bundles fail to form (Cooley et al., 1992; Verheyen and Cooley, 1994).

5.3.3. Nurse cell bundles and apoptosis

Very little is known about the pathway leading to actin bundle assembly in the nurse cells. For some time, it has been thought that an apoptotic signal induces the formation of actin bundles, because bundles do not form in a mutant lacking the caspase Dcp-1 (McCall and Steller, 1998). However, it was later shown that the mutation also affected the neighbouring gene *pita*. A subsequent analysis of double and single mutants revealed that loss of *pita* caused egg chamber degeneration during mid-oogenesis, but because Dcp-1 was also missing, the nurse cells did not die. It was suggested that the dumpless stage 10 egg chambers were arrested at a stage prior to dumping. Loss of *dcp-1* alone did not affect egg chamber development (Laundrie et al., 2003). Loss of the DP subunit of the E2F transcription factor resulted in a reduced density of nurse cell actin bundles and suppressed nurse cell apoptosis (Myster et al., 2000). Premature bundle formation and nurse cell apoptosis were observed in egg chambers with a defect in acylglycerol lipid metabolism (Buszczak et al., 2002). Thus, although nurse cell death and actin bundle formation clearly are linked in time, evidence for an apoptotic signal as an inducer of actin bundle formation at stage 10B is yet to be presented (McCall, 2004).

5.3.4. Dumpless mutants with actin bundle defects

In addition to those mentioned above, a number of dumpless mutants have been described as having defects in or loss of nurse cell actin bundles (Table 4). How the corresponding gene products eventually contribute to the formation of actin bundles is not known.

5.4. The follicle cell actin cytoskeleton

The single-layered follicular epithelium that surrounds the germline cells is polarised, with its apical side facing the germline and the basal side facing the epithelial sheath that

Table 4. Genes required for nurse cell actin bundles (not in the text)

Gene	Protein product and/or function	Type of mutation	Phenotype	Reference
Dcdc42	Rho-family GTPase	Expression of dominant negative Dcdc42 / loss-of-function mutation	Loss of bundles, cortical contraction occurs / slightly disorganised bundles	Murphy and Montell, 1996 Genova et al., 2000
Drac1	Rho-family GTPase	Expression of dominant negative Drac1	Loss of bundles, cortical contraction occurs	Murphy and Montell, 1996
RhoL	Rho-family GTPase	Expression of dominant negative RhoL	Loss of bundles, cortical contraction occurs	Murphy and Montell, 1996
armadillo	β-catenin; cell-cell adhesion	Loss-of-function	Loss of bundles, cytoskeletal disruption	Peifer et al., 1993
saxophone	Receptor for Dpp	Expression of dominant negative Sax	Loss of bundles	Twombly et al., 1996
Gprk2	G protein-coupled receptor	Female sterile mutation	Unstable bundles because of low cAMP levels	Lannutti and Schneider, 2001
Datx2	Homologue of the spinocerebellar ataxia 2 (SCA2) gene product; putative regulator of RNA metabolism	Hypomorphic mutation	No bundles, cortical contraction occurs	Satterfield et al., 2002
lark	RNA-binding protein	Null mutation	Loss of bundles	McNeil et al., 2004
ovarian tumor	Component of a hnRNP complex	"Differentiated" class mutations	Loss of cytoplasmic bundles, accumulation of bundles on the nuclear surface, cortical contraction occurs	Storto and King, 1988 Rodesch et al., 1997 Goodrich et al., 2004

surrounds the ovariole. The apical side of the follicle cells extends numerous actin-based microvilli (D'Alterio et al., 2005). Actin-rich adherens junctions near the apical surface link the follicle cells to one another (Baum and Perrimon, 2001), and a layer of stress fibre-like actin bundles cover the basal side of the follicle cells (Gutzeit, 1990). Nurse cell dumping leads to a rapid increase of the oocyte volume and a concomitant expansion of the follicular epithelium. It is thought that the basal actin fibres together with the laminin fibres in the basement membrane are involved in shaping the oocyte during egg elongation (Horne-Badovinac and Bilder, 2005).

5.4.1. Organisation of the basal actin fibres

The follicle cells acquire their layer of basal actin fibres concomitantly with egg chamber assembly in the germarium. Initially, the basal actin fibres are organised perpendicularly to the A/P axis of the ovariole, but the polarity is lost when the egg chamber buds off the germarium. By stage 7, the fibres become gradually aligned perpendicularly to the A/P axis again in a process that is initiated around the polar cells. It was suggested that a planar polarity signal from the polar cells induces realignment of the actin fibres (Frydman and Spradling, 2001). During midoogenesis, the follicle cells also display a circular polarity, in that strongly stained F-actin protrusions extend from the side of the cell in one direction only. At stage 10, the fibre density increases in the follicle cells covering the oocyte, whereas in the stretch follicle cells that cover the nurse cells, the parallel fibres are transformed into a network with no axial orientation. The parallel alignment is less evident during nurse cell dumping, but it is resumed by stage 13. By late stage 14, the basal actin fibres have disappeared. Blocking actin dynamics with cytochalasin resulted in a complete loss of actin fibres (Gutzeit, 1990, 1992), indicating that they are dynamic. Laminin is a major component of the basement membrane that lines the follicular epithelium. It is organised in stripes that are co-aligned with the basal actin fibres both in the wild type and in a mutant with mixed actin fibre polarity, indicating that these two compartments communicate (Gutzeit et al., 1991). Proteins that have been shown to associate with the termini of the basal actin fibres are β-integrin, the receptor tyrosine phosphatase Dlar and Enabled (Ena) (Bateman et al., 2001; Baum and Perrimon, 2001). Ena was originally identified as a dominant suppressor of the lethality caused by mutations in the *Drosophila* Abelson tyrosine kinase, and it is the founding member of the Ena/VASP protein family. Ena/VASP proteins localise at the leading edge of lamellipodia, in the tips of filopodia, at cell-cell and cell-matrix adhesions and periodically along stress fibres. In mammalian cells and *Drosophila* syncytial embryos, Ena/VASP proteins promote the formation of long unbranched actin filaments (Grevengoed et al., 2003; Krause et al., 2003).

5.4.2. Regulators of the basal actin fibres

Several mutants are known that produce spherical eggs. This defect has been correlated with a failure to align the basal stress fibres during mid-oogenesis. The proteins implicated in this process all seem to be involved in signalling from the ECM to the actin cytoskeleton. These include the ECM component Laminin A (Frydman and

Spradling, 2001), as well as several transmembrane proteins that may act as receptors for laminin: Dystroglycan, which connects to the cytoskeleton through Dystrophin (Deng et al., 2003), Dlar (Bateman et al., 2001; Frydman and Spradling, 2001) as well as β-integrin (encoded by *myospheroid*) and the α-integrin subunit encoded by *multiple edematous wings* (Bateman et al., 2001). Other components of the ECM may also be involved, because mutations in *inflated*, which encodes an integrin α subunit that confers specificity to ECM ligands other than Laminin A, also resulted in round eggs (Bateman et al., 2001). Cytoskeletal components reported to be required for egg elongation are Talin (encoded by *rhea*) (Bécam et al., 2005) and Tensin (encoded by *blistery*) (Lee et al., 2003). Both of these directly link integrins to F-actin. RhoA has also been implicated in egg elongation (Bateman et al., 2001). For several of these mutants (*dystroglycan*, *Dlar*, *myospheroid*), it was demonstrated that the polarity defect extended beyond the mutant clone. Accordingly, it was proposed that planar polarity is communicated between neighbouring cells via the ECM (Bateman et al., 2001; Deng et al., 2003). However, in the case of *Dlar*, some of the mutant clones did not show the polarity defect. As an explanation for this, it was suggested that Dlar is involved in polar cell determination, and that the polarity defect was a secondary consequence of defective signalling from the polar cells (Frydman and Spradling, 2001). The density of the basal layer of actin fibres was reduced in follicle cells lacking the transcription factor Serum response factor SRF or its cofactor MAL-D (Somogyi and Rørth, 2004a).

AIMS OF THE STUDY

This thesis addresses the roles of two actin-binding proteins, twinfilin and α-actinin, in *Drosophila* development.

Twinfilin is a recently discovered protein, and its role in actin dynamics is not yet fully understood. Because it is an evolutionarily conserved protein, yet not required for viability in yeast, *Drosophila* was chosen as a model organism in order to clarify its role in a multicellular organism. The analysis was aided by the identification of a P-element line with an insertion in the *twinfilin* gene. The specific aims were:

1. To clone the *Drosophila twinfilin* gene, analyse its expression and investigate the distribution of the Twinfilin protein in *Drosophila* tissues.
2. To analyse *twinfilin* expression in the P-element line and characterise the bristle phenotype resulting from the insertion.
3. To generate UAS-*twf* transgenic flies and perform rescue and overexpression experiments.

α-Actinin has been extensively studied in cell culture systems, while there is considerable less knowledge about its role in vivo. In *Drosophila*, the only identified role for α-actinin is in the organisation of the muscle sarcomere. A P-element inserted in an untranslated exon specific for the non-muscle α-actinin isoform provided an opportunity to gain knowledge about the contribution of this particular isoform to *Drosophila* development. The specific aims were:

1. To generate an α-*actinin* mutant specifically lacking the non-muscle isoform.
2. To analyse α-actinin expression in wild type and mutant animals.
3. To identify a mutant phenotype resulting from lack of α-actinin in a non-muscle tissue.

MATERIALS AND METHODS

Table 5. Methods used in this study

Method	Used in and/or Reference
Molecular methods	
Cosmid library screening by colony hybridisation	II
Subcloning	II
Restriction enzyme mapping	II
Southern blot	II
Northern blot	I, II
RT-PCR	II, III
Single-fly PCR	Gloor et al., 1993
Examination of tissues	
Immunolocalisation of proteins in tissues	I, II, III
Phalloidin staining of F-actin	I, II, III
Nuclear staining with Hoechst 33258 or propidium iodide	I, II,III
mRNA in situ hybridisation	III
Conventional and confocal microscopy	I, II, III
Scanning electron microscopy	I
Fly methods	
Crossings and recovery of recombinant chromosomes	I, II, III, unpublished data
P-element excision mutagenesis	II, unpublished data
Generation of somatic clones using the FLP-FRT system	II, III; Xu and Rubin, 1993
Generation of germline clones using the FLP-FRT female sterile technique	Chou and Perrimon, 1992
Overexpression using the UAS-GAL4 system	III, unpublished data; Brand and Perrimon, 1993
Colchicine feeding	III

Generation of UAS-*twf* transgenic flies (unpublished data)

The *twinfilin* cDNA (I) was cloned in the pUAST-vector and injected into *white* embryos. Seven independent insertions, which were recovered from one single transformant, were mapped to all three major chromosomes. For the rescue experiment, recombinant twf^{3701} UAS-*twf* and twf^{3701} B11-GAL4 chromosomes were generated. The presence of twf^{3701} on the recombinant chromosomes was confirmed by crossing each recombinant with the original twf^{3701} mutant. Bristles on flies with GAL4-driven *twf* expression were examined under a stereo microscope.

Table 6. Antibodies used in this study

Antigen	Code	Source and/or Reference
α-Actinin	MAC276	Lakey et al., 1990; Babraham Institute, Cambridge
Anillin		Field and Alberts, 1995; C. Field
Axons in the CNS	BP102	Developmental Studies Hybridoma Bank (DSHB)
Broad-core	25E9.D7	DSHB
Enabled	5G2	DSHB
Hts-F (fusome)	7H9 1B1	DSHB
Hts-RC (ring canals)	655 4A	Robinson et al., 1994; L. Cooley
c-Myc	9E10	Santa Cruz Biotechnology, Inc.
Quail	6B9	Mahajan-Miklos and Cooley, 1994; L. Cooley
Singed	7C	Cant et al., 1994; L. Cooley
Phosphotyrosine	PY20	Santa Cruz Biotechnology, Inc.
Twinfilin		This thesis (I)

Table 7. Fly strains used in this study

Genetic element	Relevant properties or purpose	Source
Alleles		
w; EP(3)3701/TM6Tb	P-element inserted in the *twinfilin* gene	Szeged Drosophila Stock Centre
Df(3R)su(Hw)7/TM6B, Tb^1	Deficiency uncovering the *twinfilin* gene	Bloomington Stock Center
$y^1\ w^{67c23}$; P{lacW}tsr^{k05633}/CyO	Cofilin mutant allele	Bloomington Stock Center
P{lArB}WG1118; ry^{506}	P-element inserted in the *α-actinin* gene	Walter Gehring
$Actn^3$	No adult muscle-specific α-actinin produced	Umeå Drosophila Stock Center
In(1)HC207, $Actn^8$/FM7a	*α-Actinin* null allele	Bloomington Stock Center
$Actn^{14}$/FM7a	*α-Actinin* null allele	Bloomington Stock Center
$Actn^{14}/Actn^{14}$; B22/+	Source of transgene providing ubiquitous expression of adult muscle-specific α-actinin	Ronald Dubreuil
usp^2/FM7	Insertion in the *α-actinin* gene	Ronald Evans
usp^3 hs-N-myc FRT18A; λ10 *Tb*/TM3	Source of 8 kb genomic fragment containing usp^+	Margrit Schubiger
$qua^1\ cn^1\ bw^1\ sp^1$/CyO	*quail* mutant; required for nurse cell actin bundles	Bloomington Stock Center
In(1)Mud, Mud^1/In(1)dl-49, sn^{X2}	*singed* mutant; required for nurse cell actin bundles and bristles	Bloomington Stock Center
w; $kel^{DE1}\ cn^1$/CyO	*kelch* mutant; occluded ring canals	Bloomington Stock Center
ru h st p^p $cher^{sko}$ *e ca*/TM6B, *D Hu h ca*	*cheerio* mutant; ring canals lacking an inner rim	Thomas Hays
UAS-lines		
UAS-*λtop* 4.4	Activated EGF receptor	Trudi Schüpbach
y1 w; P{UAS-*Egfr*.DN.B}29-77-1; P{UAS-*Egfr*.DN.B}29-8-1	Dominant negative EGF receptor	Bloomington Stock Center
w; P{UAS-*dpp*.S}42B.4	Ectopic Decapentaplegic (Dpp)	Bloomington Stock Center
GAL4-lines		
w; E4-GAL4	Posterior follicle cells	Trudi Schüpbach
P{GawB}T155 (T155-GAL4)	All follicle cells from stage 9	Bloomington Stock Center

w; GR1-GAL4	All follicle cells	Trudi Schüpbach
w; CY2-GAL4	All follicle cells from stage 8	Trudi Schüpbach
w; P{GawB}55B (55B-GAL4)	Anterior follicle cells	Bloomington Stock Center
B11-GAL4/TM6BcTb	Bristles	Kathryn Miller
FLP-FRT mediated recombination		
w^{1118} P{neoFRT}18A	Generation of FRT18A recombinant chromosomes	Bloomington Stock Center
w^{1118} P{πM}10D P{neoFRT}18A	FRT18A chromosome marked with a heat shock-inducible nuclear Myc-tag	Bloomington Stock Center
y^1 *w* v^{24} P{FRT(w^{hs})}101	Generation of FRT101 recombinant chromosomes	Bloomington Stock Center
y w Ubi-GFP FRT101	FRT101 chromosome marked with nuclear GFP	François Payre
w ovo^{D1} v^{24} P{FRT(w^{hs})}101/C(1)DX, y^1 f^1/Y; P{hsFLP}38	Source of hsFLP; generation of germline clones	Bloomington Stock Center
y^1 *w* v^{24} P{FRT(w^{hs})}101/FM7a; P{en2.4-GAL4}e22c P{UAS-FLP1.D}JD1/CyO	FLP-FRT mediated somatic recombination induced in the somatic stem cells of the ovary	Bloomington Stock Center
Miscellaneous strains		
Canton-S	Wild type	Bloomington Stock Center
Oregon-R	Wild type	Eliane Mohier
w^{1118}	Wild type	Jan Larsson
TM2, ry^1 P{Δ2-3}/MKRS, Sb^1 P{Δ2-3}99B	P-element excision	Umeå Drosophila Stock Center
w; Dr^1/TMS, P{Δ2-3}99B	P-element excision	Bloomington Stock Center
FM7a, *y B*/y^1; ry^{506}	Balancer stock for establishing P-element excision lines	Umeå Drosophila Stock Center
FM7i, P{ActGFP}JMR3/C(1)DX, y^1 f^1	Balancer and dominant marker	Christos Samakovlis
Df(1)NP5, *hdp*-b^{p363}/FM7c, P{act-lacZ.B}GD1 P{act-lacZ.B}GD2	Balancer and dominant marker	Bloomington Stock Center
w; In(2LR) noc^{4L} Sco^{rv9R} b^1/CyO, P{ActGFP}JMR1	Balancer and dominant markers	Christos Samakovlis
w; Cx^D/TM3SblacZ	Balancer and dominant markers	Christos Samakovlis
y; hs-*usp Ki* p^p/TM3SbSer	Source of TM3SbSer balancer and dominant marker	Ronald Evans
y^1 w^1 f^1	Recessive markers for the X-chromosome, used to generate recombinant $Actn^{\Delta 233}$ chromosomes	Bloomington Stock Center

RESULTS AND DISCUSSION

6. Twinfilin (I, unpublished data)

6.1. Identification of the *Drosophila twinfilin* gene and a *twf* mutant fly strain

The *Drosophila twinfilin* (*twf*) gene was identified in a sequence similarity search of the *Drosophila* genome. The amino acid sequence is 26% identical to yeast twinfilin and 49% identical to mouse twinfilin. Purified *Drosophila* Twinfilin bound actin monomers and prevented actin filament assembly in vitro similarly to yeast and mouse twinfilins. A fly strain with a P-element inserted in the first intron of the *twf* gene was identified, and the insertion was found to correspond to a strong hypomorphic mutant allele that was named twf^{s701}. Northern blotting, western blotting and immunostaining experiments showed that Twinfilin was ubiquitously expressed in the wild type and severely reduced in the mutant. *Twf* mutant flies exhibited reduced motility and viability, a prolonged larval period, rough eyes and severely malformed macrochaete (I). Wild type bristle morphology and full viability were restored by precise excision of the P-element in the twf^{s701} mutant line (unpublished data). The bristle phenotype was examined in detail and will be discussed below.

6.2. *Twinfilin* mutants have defective bristles

The adult *twf* mutant macrochaete were much shorter than in the wild type and often bent, split or branched. The ridge pattern was extremely irregular (I), which is indicative of failed grafting of the individual actin bundle modules during bristle development (Guild et al., 2003). Of all bristle mutants described so far, *twf* mostly resembles the *cpb* mutant, which lacks the Capping protein β subunit (Hopmann et al., 1996).

In addition to abnormal bristles, 20% of the *twf* mutant flies had a scutellar malformation. One or both posterior scutellar bristles were bent forwards over the thorax, and the edge of the scutellum along with the bristle socket was anteriorly translocated (Fig. 5B in I). The reason for this malformation was that a fusion between the scutellar bristle and a more anterior one hindered the scutellum from assuming its normal shape. A similar scutellar malformation has previously been described for the *Bristle* mutant, and it was suggested that interdigitating projections from the two bristle surfaces may have caused the bristles to adhere to each other, thereby resulting in a fusion of the two chitin layers (Lees and Waddington, 1942).

6.3. Lack of Twinfilin disrupts actin bundle organisation in developing bristles

In order to learn how Twinfilin regulates actin bundle formation in the developing bristle, wild type and *twf* mutant pupal bristles were stained with phalloidin and examined at four time points: 32 hours, 41 hours, 48 hours and 54 hours of pupal development.

At 32 hours of development, bristle elongation is driven by actin polymerisation at the bristle tip. Actin polymerisation also occurs between the bundles further down the shaft, and in phalloidin-stained bristles this is seen as fluorescent F-actin spots between the bundles. The additional actin filaments that are added to the bundle modules as they increase in diameter (Tilney et al., 1996a; Guild et al., 2002, 2003) are probably generated at these locations. At this stage, Twinfilin protein was diffusely localised in the centre of the bristle shaft and as discrete spots between the main bundles, consistent with its role as a regulator of actin dynamics (I). The *twf* mutant bristles did not significantly differ from the wild type at this stage (not shown).

By 41 hours of development, the bristle has reached its maximal width but is still elongating (Tilney et al., 1996a, 2000b). In wild type bristles at this stage, only smooth actin bundles are visible. In contrast, *twf* mutant bristles continued to display F-actin spots or tiny bundles between the main bundles (I). F-actin spots also appear when elongating bristles are cultured in the presence of a drug that stabilises actin filaments and thereby prevents them from undergoing turnover (Tilney et al., 2003). Since Twinfilin does not bind actin filaments, the accumulation of F-actin is a result of a net rate of actin polymerisation that is higher than in the wild type. In the absence of Twinfilin's actin monomer sequestering activity, there is an increase in the pool of actin monomers available for actin filament assembly, and the rate of filament disassembly is not sufficient to balance the higher rate of polymerisation. The reason why the ectopic actin filaments remain at what apparently is their site of synthesis instead of being incorporated with the main bundles may be that the limiting bundling protein Forked has been exhausted (Tilney et al., 2000a). If this is true, an increased amount of Forked in the *twf* mutant background should eliminate the ectopic F-actin spots.

In 41 and 48 hour old *twf* mutant bristles, the main bundles also appeared very disorganised. Thick bundles were seen in the centre of the bristle, while thinner bundles twisted around the periphery (I). An increased number of bundles, which may be a result of bundle splitting, appears to be a general consequence of alterations in actin dynamics (Verheyen and Cooley, 1994; Hopmann et al., 1996; Guild et al., 2002; Hudson and Cooley, 2002a; Hopmann and Miller, 2003). In contrast, displacement of the bundles from the cell membrane was observed only under conditions that increased the amount of F-actin in the bristle (Hopmann and Miller, 2003). The fact that bundle displacement could be observed in *twf* mutant bristles thus constitutes further support for the hypothesis that loss of Twinfilin increases the level of F-actin in developing bristles.

By 48 hours of development, bundle breakdown is under way in wild type bristles. This is seen as small gaps between the bundle modules (Tilney 1996a; Guild 2002). At this stage, Twinfilin was localised at the barbed end of the bundles (I), possibly through an interaction with Capping protein (Hopmann et al., 1996; Palmgren et al., 2001; Vartiainen et al., 2003). Bundle breakdown was not perturbed by the loss of Twinfilin, since gaps between the bundle modules were observed also in the *twf* mutant. However, mutant bundles may break down slower than in the wild type, since 48 hour and 54 hour old mutant bristles appeared quite similar, whereas in the wild type, bundle breakdown

had clearly advanced in the older bristles compared to the younger (not shown). Since module shortening occurs at the barbed end (Guild et al., 2002), where Twinfilin can be detected, Twinfilin may have a role in bundle disassembly at the barbed end. Capping protein is also thought to be localised at the barbed end (Hopmann et al., 1996; Hopmann and Miller, 2003). Since Capping protein bound to an actin filament prevents monomer loss, some mechanism that removes Capping protein should be activated before module shortening can begin. It is, however, unlikely that Twinfilin would antagonise Capping protein, because neither yeast nor mouse twinfilin had any effect on the activity of capping protein in vitro (Falck et al., 2004). A more likely scenario is that Twinfilin localised at the barbed end during bundle disassembly sequesters the released monomers, thereby preventing them from being reassembled onto the barbed end.

6.4. *Twinfilin* interacts genetically with *twinstar*

In yeast, lack of twinfilin reinforced the effect of a temperature-sensitive cofilin allele, resulting in lethality at the permissive temperature. This was suggested to be due to a synergistic depletion of the actin monomer pool (Goode et al., 1998). A genetic interaction between *twf* and *twinstar* (*tsr*), the gene encoding *Drosophila* Cofilin, was detected also in the fly, in that doubly heterozygous flies displayed a weak bristle phenotype (I). Yeast cofilin limits F-actin assembly by increasing the rate of actin depolymerisation (Paavilainen et al., 2004). In agreement with this, *Drosophila tsr* mutant cells have higher levels of F-actin (Chen et al., 2001). Thus, the observed genetic interaction supports the concept that Twinfilin acts to limit F-actin assembly.

6.5. Overexpression of *twinfilin* rescues the *twf* bristle phenotype (unpublished data)

Transgenic UAS-*twf* flies were generated in order to perform rescue experiments and to study the effect of overexpression of Twinfilin. The bristle defect in the twf^{3701} mutant was fully rescued when *twf* expression was driven in developing bristles. This confirms that functional Twinfilin protein is made from the used UAS-*twf* construct and proves that the severely reduced level of Twinfilin is the cause of the observed bristle defects in the twf^{3701} mutant.

Overexpression of *twf* in wild type bristles did not result in detectable defects. The lack of an effect could be an indication of the unlimited amount of G-actin available in the elongating bristle (Tilney et al., 2000b). The additional sequestering activity resulting from overexpression of Twinfilin may therefore not be sufficient to diminish the G-actin pool below a threshold level required for normal bundle formation. Alternatively, if Twinfilin needs a partner (e.g. Capping protein or some activating enzyme) in order to perform its function in the cell, a limited availability of that partner could prevent excess Twinfilin from altering actin dynamics.

7. α-Actinin

7.1. Generation of novel α-*actinin* mutant alleles (II, III)

The *Drosophila* α-*actinin* (*Actn*) gene (Fig. 7) gives rise to three different α-actinin isoforms through alternative splicing (Roulier et al., 1992). In addition, different promoters are utilised for the non-muscle and muscle-specific transcripts (Fyrberg et al., 1998). In the fly strain WG1118, a P-element is inserted in the first untranslated exon of the non-muscle α-actinin transcript. Excision of the P-element resulted in the recovery of three novel non-muscle-specific *Actn* mutant alleles: the viable and fertile $Actn^{\Delta 233}$ and the two lethal alleles $Actn^{\Delta 208}$ and $Actn^{\Delta 148}$. The *ultraspiracle* insertion allele usp^2 (Oro et al., 1990; Schubiger and Truman, 2000) constitutes a fourth non-muscle-specific *Actn* mutant allele. The lethality of $Actn^{\Delta 148}$ could be rescued by a transgene providing wild type Usp function. A Northern blot analysis showed that the level of non-muscle α-actinin mRNA was severely reduced in the $Actn^{\Delta 233}$ mutant. However, in an RT-PCR analysis, spliced mRNA containing the internal non-muscle-specific exon was still detected in both $Actn^{\Delta 233}$ and $Actn^{\Delta 148}$, suggesting that non-muscle α-actinin can be transcribed from a second promoter in addition to the previously identified.

7.2. α-Actinin expression during embryogenesis (II)

According to the Northern blot analysis, only embryos express high levels of non-muscle-specific α-actinin mRNA. Immunolocalisation of α-actinin protein in wild

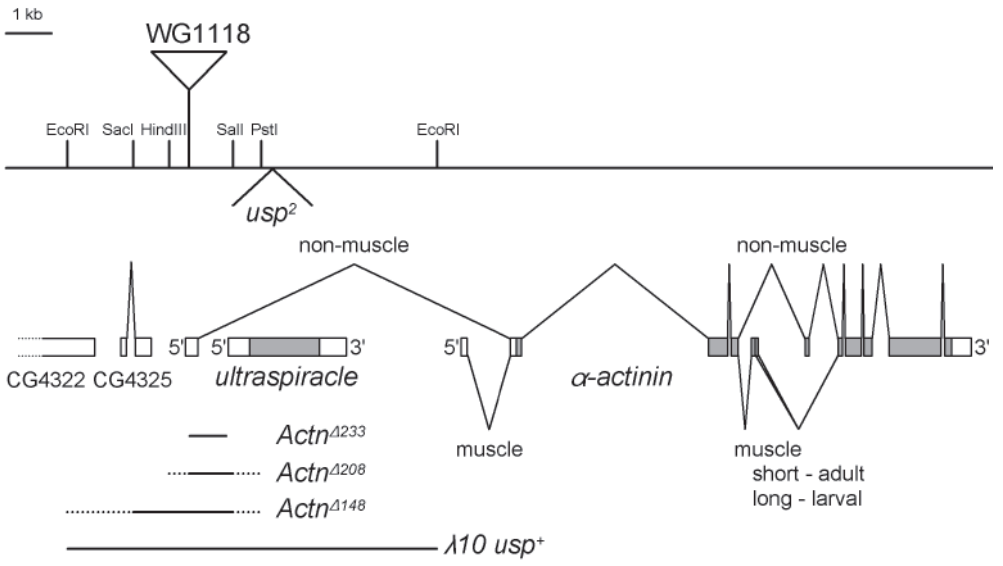

Figure 7. Organisation of the α-*actinin* locus. The *Actn* deletions and the usp^+ rescue fragment are shown, as well as the insertion site in the usp^2 allele.

type embryos, which was carried out using an antibody recognising all three isoforms, revealed a ubiquitous high expression. The axons of the central nervous system (CNS) were also outlined. In *Actn*$^{\Delta 233}$ mutant embryos, α-actinin was detected in muscle tissues as expected, but also in the denticle belt primordia (II) as well as more widely in the epidermis of older embryos (not shown). No α-actinin was present in young mutant embryos nor in the CNS at any stage of embryogenesis. Thus, non-muscle α-actinin in young embryos and in the CNS is produced from an mRNA containing the first untranslated exon. In contrast, at least part of the epidermal α-actinin is expressed from an mRNA not dependent on this exon. The fact that embryogenesis proceeded normally in the *Actn*$^{\Delta 233}$ mutant is in agreement with a previous study showing that *Actn* null mutant embryos derived from germline clones hatch (Perrimon et al., 1985).

7.3. α-Actinin protein expression during oogenesis - novel events revealed (II, III, unpublished data)

In wild type egg chambers, α-actinin was ubiquitously expressed. In *Actn*$^{\Delta 233}$ mutant egg chambers, α-actinin was absent from some cell populations but present in others. Thus, like in the embryo, at least two differently regulated populations of α-actinin are expressed in the egg chamber (Table 8). The same expression patterns were seen also in egg chambers from homozygous *Actn*$^{\Delta 148}$ and *usp*^{2} females rescued with the *usp*$^{+}$ transgene. Considering that the insertion in *usp*^{2} is over 1 Mb in size, this population of α-actinin must be expressed from a promoter that is located downstream of *usp*, possibly the muscle-specific promoter. To avoid making an untrue statement regarding the type of isoform, this particular population of α-actinin is referred to as FC-α-actinin (for Follicle Cell).

Table 8. Summary of α-actinin protein expression patterns in the ovary

Structures and cells containing only non-muscle α-actinin	**Structures and cells containing FC-α-actinin**
Junction between the cap cells and the germline stem cells	Polar cells
Arrested cleavage furrows between cystocytes	Apical follicle cell borders in degenerating egg chambers
Follicle cells at mid-oogenesis, cortically and basal actin fibres	Main body follicle cells starting from stage 10, cortically and basal actin fibres
Dorsal anterior follicle cell, cortically and basal actin fibres	Occasional punctated staining in the follicle cells covering the nurse cells
Vacuoles in follicle cells of degenerating egg chambers	
Oocyte cortex	
Nurse cell cortex	
Ring canal inner rim starting from stage 10	
Nurse cell actin bundles	

7.3.1. Non-muscle α*-actinin in the germarium* (unpublished data)

In the germarium, an accumulation of α-actinin was seen in the junction between the somatic cap cells and the germline stem cells (Fig. 8A). The cap cells are connected to the germline stem cells through adherens junctions containing DE-cadherin and the β-catenin homologue Armadillo, and this contact is necessary to anchor the germline stem cells in their niches (Song et al., 2002). α-Actinin may be a component of this junctional adhesion complex, since mammalian α-actinin is known to associate with adherens junctions via α-catenin (Knudsen et al., 1995; Nieset et al., 1997). An enrichment of α-actinin was also detected in region 1 of the germarium in the vicinity of the fusome. This pattern most likely represents the arrested cleavage furrows between the cystocytes (Fig. 8B). These staining patterns were absent from the $Actn^{\Delta 233}$ mutant germaria (Fig. 8C), they are thus derived from expression of non-muscle α-actinin.

7.3.2. α*-Actinin in apoptotic egg chambers* (unpublished data)

Ovaries from $Actn^{\Delta 208}/Actn^{\Delta 233}$ transheterozygotes (but not homozygotes from the *y* $Actn^{\Delta 233}$ *w* recombinant stock) frequently contained stage 8-9 degenerating egg chambers. In such egg chambers, FC-α-actinin was detected at the apical surface of the follicle cells, which appeared to contract around the shrinking nurse cell compartment. In wild type degenerating egg chambers, the apical border was also evident, but in addition, intense α-actinin-positive patches were present in the follicle cells (Fig. 9). It has been shown that the apoptotic nurse cells are phagocytosed by the follicle cells, and that electron-dense vacuoles appear in the follicle cells during this process (Giorgi and Deri, 1976; McCall, 2004). The α-actinin-positive patches could correspond to such vacuoles containing non-muscle α-actinin released from the degraded nurse cell actin cytoskeleton.

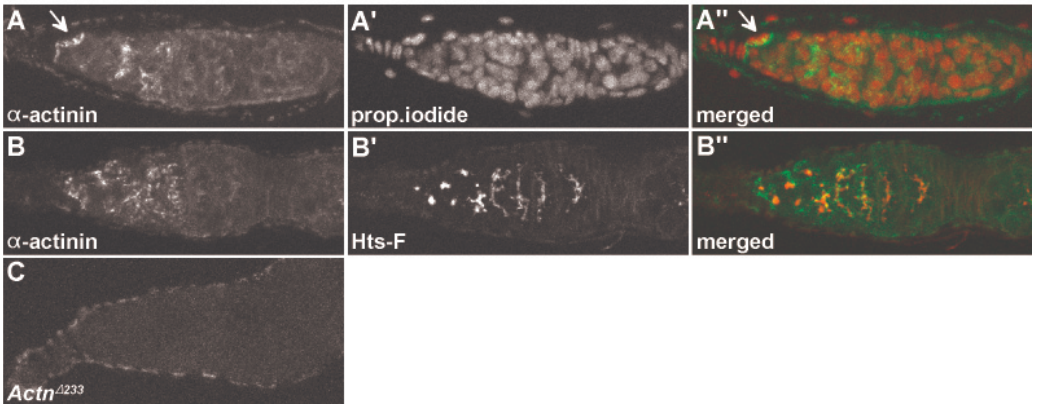

Figure 8. (A,B) Wild type germaria. (A) α-Actinin at the border between the cap cells and the germline stem cells (arrow). Propidium iodide labels the nuclei. (B) α-Actinin accumulation in the vicinity of the fusome, which is labelled with anti-Hts-F. (C) An $Actn^{\Delta 233}$ mutant germarium stained with anti-α-actinin. α-Actinin is present only in the muscular epithelial sheath that surrounds the ovariole.

Since FC-α-actinin was never detected in $Actn^{\Delta 208}/Actn^{\Delta 233}$ healthy egg chambers at stage 9, its appearance may indicate that egg chamber degeneration induces de novo gene expression in the follicle cells. Alternatively, the follicle cells may continue at least some aspects of their developmental programme, which in this case would include initiation of FC-α-actinin expression, even though egg chamber development has arrested.

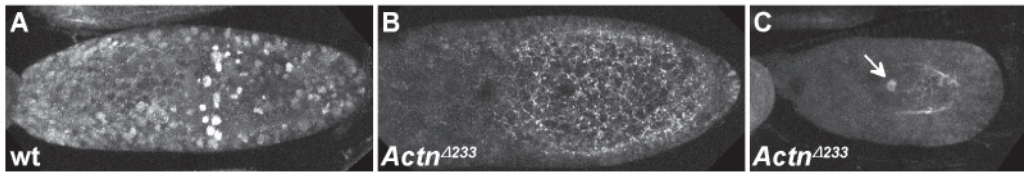

Figure 9. Apoptotic egg chambers stained with anti-α-actinin. Large patches stain in the wild type (A), whereas in the $Actn^{\Delta 233}$ mutant (surface in B, cross-section in C), only the apical follicle cell borders are stained. The arrow marks the FC-α-actinin-positive polar cells.

7.3.3. Non-muscle α*-actinin in the nurse cell actin bundles* (II, unpublished data)

The nurse cell actin bundles were only faintly stained with the anti-α-actinin antibody (II). Because the GdnHCl-treatment necessary for detection of α-actinin did not allow simultaneous labelling with phalloidin, the integrity of the actin bundles was checked using anti-Quail. This is the only known antibody that labels the nurse cell actin bundles (Mahajan-Miklos and Cooley, 1994). Surprisingly, the GdnHCl-treatment greatly improved the detectability of Quail. The nurse cell actin bundles in stage 10B egg chambers were evenly and strongly stained, in contrast to the punctated staining pattern seen in the absence of GdnHCl (Fig. 10). The dots overlapped with the weakly fluorescent sites on the phalloidin-stained F-actin bundles. These sites are thought to correspond to sites with fewer actin filaments arranged in parallel (Riparbelli and Callainin, 1995; Guild et

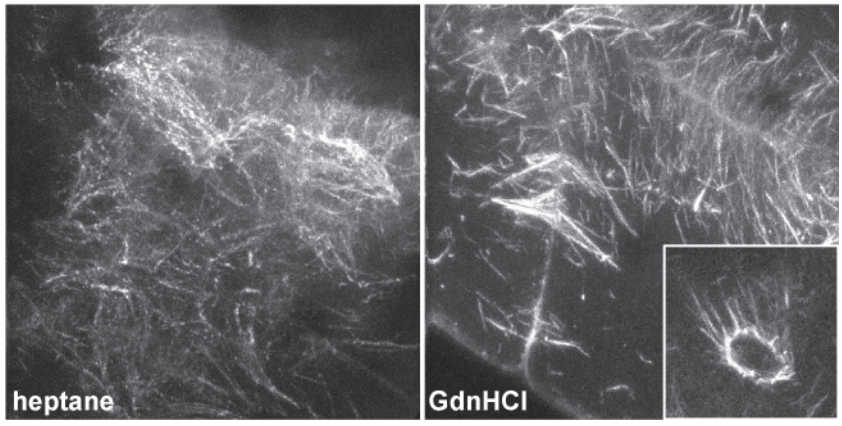

Figure 10. Punctated anti-Quail staining of the actin bundles in an egg chamber fixed with PFA and heptane, even staining in a PFA-fixed and GdnHCl-treated egg chamber. Inset: Dense Quail-containing bundles surrounding a ring canal.

al., 1997), and they are apparently more easily accessed by the anti-Quail antibody in the absence of GdnHCl. While enhancing the detectability of Quail, the GdnHCl-treatment still did not allow detection of Fascin (not shown), the second crosslinking protein present in the nurse cell actin bundles (Cant et al., 1994). Thus, the GdnHCl-treatment has no negative impact on the nurse cell actin bundles. The faint anti-α-actinin signal could therefore be due to mostly hidden epitopes, as apparently is the case for Fascin. Alternatively, α-actinin is only weakly associated with the nurse cell actin bundles.

All three actin bundling proteins, α-actinin, Quail and Fascin, were detected in the thick actin bundles that surround and traverse the degenerating nurse cell nuclei at stage 13 (Fig. 11). At late stage 13, large α-actinin- or Fascin-positive lumps resembling nurse cell nuclei were also seen. Hoechst or propidium iodide labelling gave a very variable nuclear staining at this stage, ranging from very intense to barely visible. Strongly staining nuclei were associated with distinct α-actinin-positive fibres. α-Actinin-positive nuclei, on the other hand, stained weakly or not at all with propidium iodide (Fig. 11A). It has been shown that the actin bundles assume an asymmetric arrangement around the nuclei at stage 12 and traverse the fragmenting nuclei at stage 13 (Nezis et al., 2000), but whether the bundles are actively involved in the degradation of the chromatin is not known. Phalloidin never showed nuclear-like staining, indicating that F-actin was degraded simultaneously with the chromatin. This process presumably releases α-actinin and Fascin, which remain intact for some time in the nuclear space.

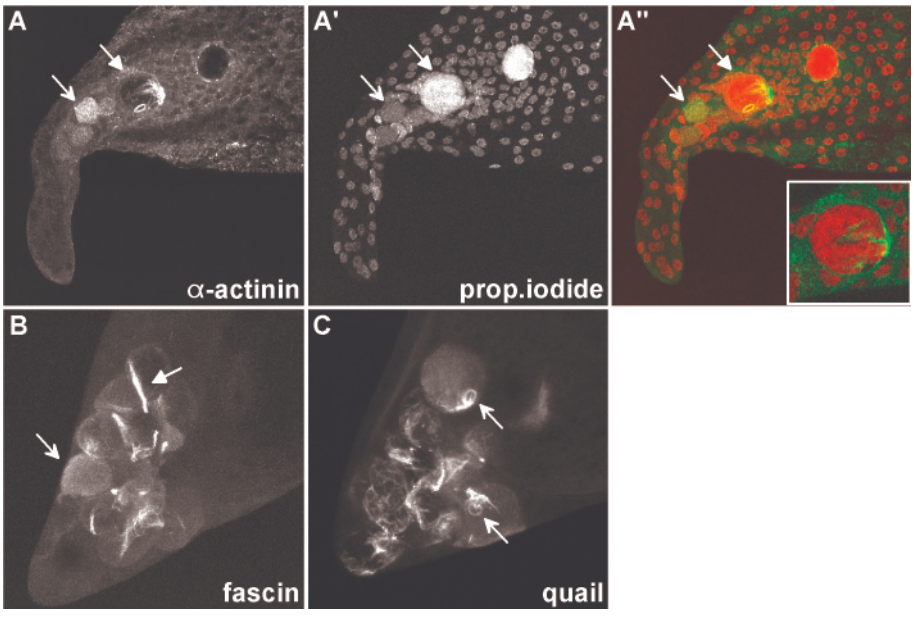

Figure 11. Stage 13 egg chambers. (A) Strongly staining nuclei are traversed by α-actinin-containing fibres (closed arrow, inset), whereas weakly stained nuclei contain α-actinin throughout (open arrow). (B) Fascin in the actin bundles (closed arrow) and degenerating nuclei (open arrow). (C) The arrows mark two Quail-positive ring canals.

7.3.4. Non-muscle α*-actinin in the ring canals* (II, unpublished data)

The nurse cell cortical actin cytoskeleton appeared denser around the ring canals than elsewhere in the cell, and accordingly, also α-actinin was enriched around the ring canals. Only from stage 10 onwards was it possible to unambiguously detect α-actinin in the ring canal itself. Examination of α-actinin localisation in *kel*^{DE1} and *cher*^{sko} mutant ring canals revealed that α-actinin was specifically localised in the ring canal inner rim (II). Anillin was also detected in the ring canals from stage 11 onwards (Fig. 12). Anillin is recruited to the arrested cleavage furrows in the germarium and persists on the ring canal outer rims until stage 2 (Field and Alberts, 1995; de Cuevas and Spradling, 1998). The appearance of α-actinin and reappearance of Anillin in late stage ring canals may be related to the reorganisation of the F-actin in the inner rim that takes place at stage 9, when the loosely organised F-actin ring becomes separated into discrete bundles (Tilney et al., 1996b). The epitopes recognised by anti-α-actinin and anti-Anillin may not be accessible at earlier stages, or alternatively, the reorganisation of the inner rim involves recruitment of additional ring canal components.

During stage 13, the α-actinin staining on the ring canals was more intense than at earlier stages, and the α-actinin-positive ring canals did not always stain positive for the ring canal marker Hts-RC (II). Furthermore, Quail was also detected on the ring canals at this stage (Fig. 11C). By contrast, at stage 10, Quail was apparently only localised in the F-actin-rich cortex around the ring canal, not in the ring canal itself (Fig. 10, inset). Thus, by stage 13, the ring canals appear to have undergone yet some changes. A possible explanation is that the subcortical F-actin network that surrounds the ring canal at stage 10 condenses and forms an outer ring by stage 13. This would explain both the increased staining of α-actinin and the appearance of Quail on the stage 13 ring canals. If the ring canal itself detaches from this outer condensed ring, the outcome would be rings that stain for α-actinin but not Hts-RC, as was observed.

7.3.5. Cytoskeletal remodelling in the main body follicle cells (III)

In the follicle cells, α-actinin is localised both at the cell cortex and in the basal actin fibres. Only non-muscle α-actinin is expressed prior to stage 10. From stage 10 onwards, the main body follicle cells express both non-muscle α-actinin and FC-α-actinin, whereas the dorsal anterior follicle cells express only non-muscle α-actinin.

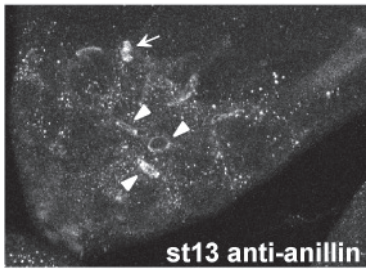

Figure 12. Stage 13 ring canals (arrowheads) stained with anti-anillin. The arrow marks the Anillin-positive polar cells.

The basal α-actinin pattern in the main body follicle cells exhibited a dynamic behaviour during stages 10 to 13. At stage 10, α-actinin was localised in the parallel actin fibres. During stages 11 and 12, α-actinin accumulated in a posterior patch, which at stage 12/13 gradually resolved into two lateral stripes. Enabled (Ena) and the common β-integrin subunit encoded by *myospheroid* (*mys*) were previously shown to localise similarly to α-actinin in stage 13 follicle cells, indicating that the lateral stripes correspond to cell-matrix adhesion sites (Bateman et al., 2001; Baum and Perrimon, 2001). Since Ena and α-actinin colocalised also in the posterior patch at stages 11 and 12, it probably represents a transient adhesion site.

The oocyte increases dramatically in volume during nurse cell dumping, and this is accompanied by flattening and elongation of the overlying main body follicle cells. The actin cytoskeleton must obviously reorganise during this process. The apparently only previous observation indicating some kind of remodelling is the one made by Gutzeit (1990), who noted a "temporary disturbance" in the basal actin fibre pattern. This was indeed the case, but cells that clearly had reoriented their basal actin fibres in a manner corresponding to the pattern observed in egg chambers stained with anti-α-actinin or anti-Ena were also identified. Thus, the main body follicle cells expand their surfaces in an organised and asymmetric manner during egg elongation. This organised remodelling may be the result of a series of signalling events initiated earlier, for example at the induction of follicle cell migration at stage 9 (Horne-Badovinac and Bilder, 2005), which then culminate in the remodelling of the basal cytoskeleton. A second more interesting possibility is that the mechanical stretch experienced by the follicle cells during nurse cell dumping induces the cytoskeletal remodelling.

Mechanotransduction may occur via two different mechanisms. One involves stretch-activated transmembrane ion channels. The other relies on conformational changes within cell-matrix or cell-cell adhesion sites that are triggered by mechanical stress (Lee et al., 1999; Geiger and Bershadsky, 2002; Tamada et al., 2004; Tzima et al., 2005). In the egg chamber, mechanical stretch was shown to induce nuclear accumulation of MAL-D in migrating border cells. MAL-D is a cofactor for the transcription factor Serum response factor (SRF), and they are both required to build a robust actin cytoskeleton in the border cells and a dense layer of basal actin fibres in the columnar follicle cells (Somogyi and Rørth, 2004a). Mechanical stress can also determine stress fibre orientation. Mammalian cells exposed to cyclic stretch respond by reinforcing their stress fibres and aligning them in a direction perpendicular to the direction of stretch. Another type of mechanical stress, fluid shear stress, instead results in an alignment that is parallel to the direction of the force. In both cases, zyxin was mobilised to the stress fibres in an integrin-dependent manner and necessary for stress fibre enforcement (Yoshigi et al., 2005). The Rho GTPase pathway and the formin mDia are involved in determining the direction of alignment. If either one of them was inhibited, the stress fibres assumed a parallel instead of a perpendicular alignment in response to stretch (Kaunas et al., 2005).

It has been assumed that the parallel basal actin fibres in the main body follicle cells help shaping the egg chamber by preventing axial expansion (Horne-Badovinac and

Bilder, 2005). This model may well apply for egg chamber growth during stages 7-9, but is difficult to reconcile with the fact that the fibres temporarily repolarise during egg elongation. Thus, rather than only restricting axial growth, the actin fibres may also be involved in directing growth during egg elongation.

7.4. Non-muscle α-actinin is not required for oogenesis (II, III)

The overall appearances of germaria, egg chambers and mature eggs were normal in females lacking non-muscle α-actinin. The nurse cell bundles in non-muscle α-actinin deficient egg chambers were examined using phalloidin, and the ring canals were analysed using the anti-Hts-RC antibody, but no abnormalities were detected. Furthermore, no genetic interactions were detected between $Actn^{\Delta 233}$ and *quail*, *singed*, *cheerio* or *kelch*. Thus, α-actinin's actin crosslinking activity does not appear to critically contribute to the stability of the various F-actin structures in the nurse cells. The F-actin pattern in the dorsoanterior stage 10B-11 follicle cells and in elongating dorsal appendages was also examined, but no obvious differences between the wild type and the mutant were observed. However, Ena was diffusely localised in mutant dorsal appendage cells, whereas it appeared in basal accumulations in the wild type. See also Chapter 7.7.2.

7.5. Which α-actinin isoform corresponds to FC-α-actinin? (II, III)

Two promoters that give rise to α-actinin mRNAs with different 5'-UTRs have been identified. The upstream promoter is utilised for the production of non-muscle-specific α-actinin, whereas the downstream promoter gives rise to muscle-specific α-actinin (Fyrberg et al., 1998). In the non-muscle *Actn* mutants, only the downstream promoter generates detectable α-actinin protein (FC-α-actinin). According to the RT-PCR analysis, all three splice variants are expressed in $Actn^{\Delta 233}$ and $Actn^{\Delta 148}$. Thus, α-actinin mRNA spliced in the non-muscle mode can presumably also be transcribed from the downstream promoter (unless there is still a third promoter to be identified), and FC-α-actinin could therefore correspond to any of the three isoforms.

In isolated wild type egg chambers, both the non-muscle and the adult muscle-specific splice variants were detected. The larval muscle splice variant was present in whole ovaries but not in isolated egg chambers, indicating that this isoform is mainly expressed by the muscle cells of the ovary. The in situ hybridisation showed that all late stage follicle cells, including the dorsoanterior ones, express a muscle-specific mRNA. Taken together, these results suggest that in the wild type, adult muscle-specific α-actinin protein may be expressed in the late stage follicle cells in addition to the non-muscle isoform. Considering the fact that FC-α-actinin protein is absent from the dorsoanterior cells, the muscle-specific transcript may be translated only in the main body follicle cells.

In conclusion, the performed experiments could not conclusively solve the question of which α-actinin isoforms are included in FC-α-actinin, although a good guess is that it corresponds to both adult muscle-specific α-actinin and non-muscle α-actinin. To conclusively solve the question of which α-actinin isoforms are expressed in the follicle

cells, isoform-specific α-actinin antibodies need to be generated.

7.6. FC-α-actinin is negatively regulated by combined EGFR and Dpp signalling (III)

The shape of the domain lacking FC-α-actinin expression is very similar to the one that experiences Epidermal Growth Factor Receptor (EGFR) activation and acquires dorsal anterior cell fate (Peri et al., 1999). The dorsoanterior domain becomes specified at stage 10 by Gurken signalling, localised above the oocyte nucleus, to EGFR in the overlaying follicle cells. This activates the Ras-Raf-MEK-MAPK/ERK signal transduction cascade, resulting in the activation of two additional EGFR ligands, Spitz and Vein, and further amplification and lateral expansion of the EGFR signal. The highest signalling levels are reached at the dorsal midline, where the EGFR inhibitor Argos is induced. Consequently, EGFR signalling is downregulated at the dorsal midline, while being retained in two lateral patches that will give rise to the dorsal appendages (Wasserman and Freeman, 1998; Shilo, 2005). The position of the dorsal appendages on the A/P axis is regulated by Decapentaplegic (Dpp) secreted by the stretch follicle cells and the first row of the centripetally migrating columnar follicle cells (Twombly et al., 1996; Deng and Bownes, 1997). Alterations in either one of these pathways change anterior egg shell structures, and both pathways are required for induction of dorsoanterior cell fate in the posterior of the egg chamber (Peri and Roth, 2000).

To test whether EGFR signalling could directly downregulate FC-α-actinin, the ligand-independent activated EGF receptor construct λ*top* was ectopically expressed in all follicle cells in the *Actn*$^{\Delta 233}$ background. Depending on the strength of the GAL4-driver used, the anterior cells acquire either dorsal appendage fate or dorsal midline, i.e. operculum, fate (Queenan et al., 1997). Ectopic expression of λ*top* in all follicle cells in the *Actn*$^{\Delta 233}$ background inhibited FC-α-actinin expression in all anterior follicle cells, but it did not affect FC-α-actinin expression or dynamics in the main body follicle cells. This shows that EGFR signalling alone is not sufficient to block FC-α-actinin expression. Expression of a dominant negative EGFR in anterior follicle cells resulted in ventralised eggs with dorsal appendage formation only on the dorsal midline, and accordingly, the FC-α-actinin negative domain was reduced to a small patch on the dorsal midline. When both EGFR and Dpp pathways were activated in posterior follicle cells, FC-α-actinin expression was lost. Thus, lack of FC-α-actinin correlates with dorsoanterior cell fate, and both EGFR and Dpp signalling pathways are required to block FC-α-actinin expression.

7.7. α-Actinin function in main body follicle cells (III)

Since expression of FC-α-actinin in *Actn*$^{\Delta 233}$ main body follicle cells may compensate for the lack of non-muscle α-actinin, clones of cells lacking all α-actinin isoforms were generated using the somatic recombination technique and examined for possible defects. The *Actn* null mutant clones were often very large, but no defective eggs were observed, indicating that α-actinin is not required for epithelial morphogenesis. The actin cytoskeleton was examined in detail using phalloidin and anti-Ena antibodies.

7.7.1. The basal actin fibres do not depend on α-actinin function

Phalloidin staining of the mosaic egg chambers revealed that cells lacking α-actinin still possessed a basal layer of stress fibres, often indistinguishable from that in the neighbouring wild type cells. The slightly more diffuse lateral Ena-pattern in stage 13 follicle cells indicated that subtle differences nevertheless exist. However, α-actinin does not appear to play a major role in the generation or maintenance of the basal actin fibres in the follicle cells. A number of studies performed on mammalian cells in culture have suggested quite the opposite, namely that α-actinin is important for stress fibre assembly and maintenance, both as a crosslinker within the stress fibre and as a linker between the stress fibre and the cell-matrix adhesion site. In most of these experiments, a truncated α-actinin was introduced into the cell, which caused displacement of endogenous α-actinin from the adhesion sites and/or stress fibres (Pavalko and Burridge, 1991; Schultheiss et al., 1992; Hijikata et al., 1997; Pavalko et al., 1998). In another study, laser-mediated inactivation of GFP-tagged α-actinin located in an adhesion site was shown to result in stress fibre retraction (Rajfur et al., 2002). These experiments show that once α-actinin becomes localised, its further presence is necessary to maintain the stress fibre and its connection to the adhesion site. The situation is different in the *Actn* mutant follicle cells, where the actin fibres were assembled and attached to the cell membrane in the absence of α-actinin. It is possible that the basal actin fibres do not correspond to stress fibres, and therefore do not require α-actinin. It has been shown that the columnar follicle cells are able to contract during stage 10A (Gutzeit, 1992), but whether the basal actin fibres actually possess the characteristics of true stress fibres (i.e. contain myosin filaments) has not been determined (Hudson and Cooley, 2002b). Another possibility is that some other actin crosslinker can substitute for α-actinin in the basal actin fibres.

7.7.2. Altered cytoskeletal remodelling in follicle cells lacking α-actinin

Anti-Ena staining of the mosaic egg chambers revealed that there was a complete lack of posterior accumulation of Ena in the *Actn* null mutant cells at stages 11 and 12. At stages 10B/11 and 12/13, when the wild type cells were in the process of relocalising Ena, the neighbouring mutant cells showed an ordered lateral Ena pattern. This suggests that *Actn* mutant cells reorganise their basal cytoskeleton differently compared to the wild type cells during egg elongation.

One possible explanation for the lack of posterior accumulation of Ena is that α-actinin may be specifically required for recruitment of Ena to the posterior adhesion site. In cells overexpressing adult muscle-specific α-actinin, there was a reduction in the diffuse cytoplasmic Ena staining and a corresponding increase in Ena associated with F-actin (not shown), suggesting that α-actinin may actively recruit Ena to the cytoskeleton. There is no evidence for a direct interaction between α-actinin and Ena, but they share several binding partners (Krause et al., 2003; Li et al., 2003; Boukhelifa et al., 2004; Otey and Carpen, 2004). Of these, zyxin is a possible candidate for mediating the link between α-actinin and Ena. In cultured mammalian cells, Ena/VASP proteins are thought to be recruited to focal adhesions primarily, but not exclusively, by zyxin (Gertler et al.,

1996; Reinhard et al., 1996; Drees et al., 2000; Rottner et al., 2001). Several studies have shown that disruption of the interaction between α-actinin and zyxin displaces both zyxin and Mena (mammalian Ena) from focal adhesions (Drees et al., 1999; Reinhard et al., 1999; Li and Trueb, 2001; Nix et al., 2001). However, there is also evidence for the existence of an α-actinin-independent mechanism for zyxin localisation (Nix et al., 2001; Bhatt et al., 2002). An interaction between Zyxin and Ena has been detected also in *Drosophila* (Renfranz et al., 2003).

Alternatively, the posterior adhesion site may not assemble at all in the absence of α-actinin, maybe because the cells did not remodel their adhesion sites properly at the onset of egg elongation. This view is supported by the apparent delay in remodelling at stage 10B/11. Whether the cell-cell or the cell-matrix adhesion sites are the ones that are affected remains to be determined. Studies on mammalian cells have implicated α-actinin in the disassembly of both of these (Bhatt et al., 2002; Guvakova et al., 2002; Otey and Carpen, 2004; Fraley et al., 2005; Zhang et al., 2006). According to the results presented here, an adhesion site that lacks α-actinin apparently functions properly in maintaining adhesion. However, it may respond differently to signals that induce adhesion site remodelling. If mechanical stress actually would prove to be the signal that induces the reorganisation, it would assign α-actinin the role of a mechanosensitive protein.

Actn null mutant mosaic egg chambers differ in several respects from those of mutants defective in other cell-matrix adhesion site components, such as β-integrin or Dlar. *Actn* null mutant cells have normal actin fibre polarity, display cell-autonomy in the failure to remodel the actin cytoskeleton and undergo normal egg elongation. In contrast, loss of β-integrin or Dlar affects actin fibre polarity in a non-autonomous manner and blocks egg elongation (Bateman et al., 2001; Frydman and Spradling, 2001). Cross-talk between the ECM and the basal cytoskeleton is presumed to be involved in establishing the actin fibre polarity and in communicating the polarity information between the cells. These signalling events apparently do not require α-actinin. Correct actin fibre polarity in turn is important for egg elongation, whereas a proper cytoskeletal remodelling is apparently not. This raises the question of what purpose the remodelling might serve. A possibility is that the remodelling becomes important only under some conditions not prevailing in the laboratory.

7.8. Ectopic expression of adult muscle-specific α-actinin induces cytoskeletal alterations (III, unpublished data)

The transgenic fly strain B22 expresses ubiquitous and high levels of adult muscle-specific α-actinin (Dubreuil and Wang, 2000). In the egg chamber, ectopically expressed muscle-specific α-actinin localises to all F-actin structures, also those normally containing only the non-muscle isoform (II), mostly without causing any obvious cytoskeletal alterations. One exception was the dorsal appendage cells, which at stage 10B/11 developed prominent spikes rich in α-actinin, F-actin and Ena. This was seen in wild type (III), $Actn^{\Delta 233}$ and $Actn^{14}/Actn^{14}$ (not shown) backgrounds. In wild type phalloidin-stained roof cells, tiny bundles emerging from the lateral cell margins were occasionally seen

(not shown). Thus, it appears as if bundle formation would be a normal feature of the roof cells, and as if high amounts of α-actinin only increase the amount of F-actin that becomes incorporated. This could be due to the actin polymerisation-promoting effect of Ena (Krause et al., 2003), which may be recruited to the spikes by α-actinin.

Since the dorsal appendage cells are characterised by maintained EGFR signalling, the hypothesis that EGFR signalling increased α-actinin's bundling activity was tested by expressing λ*top* in the presence of the B22 transgene (Fig. 13). GR1-GAL4 drives expression of λ*top* in all follicle cells from early oogenesis, and this prevented the flattening and posterior migration of the anterior follicle cells at stage 9 (not shown). Ectopic EGFR signalling at this stage made the basal actin fibres appear sparser and more disorganised than in the wild type, but Ena showed normal punctated localisation. Ectopic expression of muscle-specific α-actinin alone resulted in an increased actin fibre density, including some abnormally thick Ena-accumulating actin fibres. When muscle-specific α-actinin was ectopically expressed in the GR1-GAL4/UAS-λ*top* background, all basal actin fibres were transformed into a single branched or unbranched F-actin bundle, into which also Ena was recruited. Although both λ*top* and B22 were co-expressed throughout oogenesis, extensive bundling of basal actin filaments was not observed before stage 9. These results show that EGFR signalling does not directly cause α-actinin to bundle F-actin. Rather, it appears to modify the activity of some other factor, which is induced independently of EGFR signalling at stage 9.

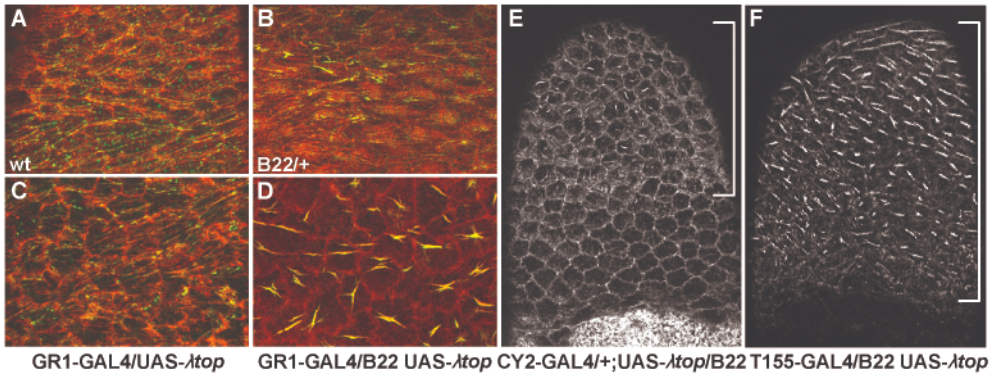

Figure 13. EGFR-signalling increases α-actinin's bundling activity. (A) Wild type stage 9 follicle cells stained with phalloidin (red) and anti-Ena (green). Ena localises as dots along the actin fibres. (B) Ectopic expression of adult muscle-specific α-actinin at stage 9. The basal actin fibres are denser, and anti-Ena reveals the presence of some thicker actin bundles as well. (C) Overexpression of λ*top* with GR1-GAL4. Ena localises normally along slightly disorganised actin fibres. (D) Overexpression of both muscle-specific α-actinin and λ*top*. All basal actin fibres have been combined into a single thick bundle. (E) Stage 10A, overexpression of muscle-specific α-actinin and λ*top* using CY2-GAL4. Only posterior cells display increased actin bundling, as visualised by phalloidin staining. (F) T155-GAL4 is used to overexpress λ*top* in the presence of ectopic muscle-specific α-actinin. Increased actin bundling occurs in all columnar follicle cells.

The relatively late expression of CY2-GAL4 and T155-GAL4 did not affect cell flattening and posterior migration during stage 9 when used to drive expression of λ*top*. With CY2-GAL4, dorsal midline fate is induced in all anterior cells. In CY2-GAL4/UAS-λ*top* B22 egg chambers, increased bundling was observed at late stage 9 and early stage 10, but only in the posterior half of the columnar epithelium. When λ*top* expression instead was driven with the weaker driver T155-GAL4, ectopic dorsal appendage material formed in the anterior. In the presence of B22, increased bundling was seen in all main body follicle cells, including the anterior ones. Thus, high levels of EGFR signalling in combination with other anterior signals do not modulate the activity of ectopic muscle-specific α-actinin, whereas high levels in the absence of anterior signals do. In contrast, medium levels of EGFR signalling increase bundling both in the presence and absence of anterior signals.

Interestingly, co-expression of muscle-specific α-actinin and λ*top* did not cause aberrant bundles in cells that normally express FC-α-actinin, i.e. main body follicle cells starting from stage 10B. The thick actin fibres present at stage 9 and early stage 10 were lost by stage 11, when the cells had reorganised their basal cytoskeleton in a normal manner (not shown). This suggests that the hypothetical bundling factor that was induced at stage 9 may be inhibited by the signals that induce the reorganisation of the basal cytoskeleton at stage 10B.

At this stage we can only speculate about the identity of the hypothetical bundling factor. In the wild type, it presumably takes part in dorsal appendage morphogenesis. The bundling factor could be a molecule that directly regulates α-actinin. Virtually nothing is known about the mechanisms that regulate the activity of *Drosophila* α-actinin. The apparently only study addressing this question demonstrated that Ca^{2+} could not bind to the EF-hands of *Drosophila* α-actinin (Dubreuil et al., 1991). Far more is known about the mechanisms that regulate the activity of vertebrate α-actinin. Mammalian α-actinin-1 phosphorylated on tyrosine 12 by FAK has a reduced F-actin binding activity (Izaguirre et al., 2001). In *Drosophila* α-actinin, the tyrosines at positions 11 and 15 are embedded within a sequence resembling that of α-actinin-1 (α-actinin-1: D**Y**MQPEEDWD; *Drosophila* α-actinin: E**Y**GDG**Y**MEQEEEWE). *Drosophila* FAK is localised basally in the follicle cells (Fox et al., 1999), where it might regulate α-actinin. An EGFR-mediated dephosphorylation of α-actinin could then account for the increased bundling activity. Phosphoinositide binding also regulates α-actinin's F-actin binding activity. Interestingly, α-actinin-1 with a reduced affinity for phosphoinositides caused increased F-actin bundling when transfected into mammalian cells (Fraley et al., 2003) in a manner resembling that of the GR1-GAL4/UAS-λ*top* B22 follicle cells. All residues required for phosphoinositide binding identified in vertebrate α-actinins (Fukami et al., 1996; Fraley et al., 2003; Franzot et al., 2005) are conserved in *Drosophila* α-actinin, indicating that the same regulatory mechanism most likely exists in *Drosophila* as well. Interestingly, two *Drosophila* genes involved in regulating the balance between the various phosphoinositides, *Pi3K92E* (encoding class I PI3K Dp110) and *Phospholipase C at 21C*, were among the genes identified in a microarray analysis as being upregulated by EGFR signalling in the follicle cells (Jordan et al., 2005).

Since α-actinin appeared to recruit Ena to the F-actin structures, it is also possible that the increased actin bundling is merely a consequence of a higher concentration of Ena being localised in the actin cytoskeleton. In that case, Ena could be the target of the EGFR pathway. How Ena activity is regulated is not yet very well understood. Phosphorylation by Abelson tyrosine kinase (Krause et al., 2003) has been shown to regulate the intracellular localisation of Ena (Grevengoed et al., 2003).

It remains to be determined whether the observed effect on the actin cytoskeleton is specific for the adult muscle-specific isoform, or if high amounts of any α-actinin isoform would cause increased bundling when inappropriately expressed. To clarify this, similar overexpression studies using non-muscle and larval muscle α-actinin should be done. In this context, it is noteworthy that larval denticle belts express FC-α-actinin (II), experience EGFR signalling (Szüts et al., 1997) and make an apical actin bundle (Dickinson and Thatcher, 1997). If the ability to respond to EGFR signalling by increased bundling activity is unique to the adult muscle-specific α-actinin isoform, and if main body follicle cells actually will prove to express this protein, the reason why FC-α-actinin is downregulated in the dorsoanterior follicle cells would be to prevent inappropriate actin bundling in cells with maintained EGFR signalling. The differences between the *Drosophila* α-actinins are in the linker region between the ABD and the first spectrin-like repeat, a region which is known to confer flexibility to the protein. A phosphoinositide bound to the ABD could interact with the linker sequence (Franzot et al., 2005) and thereby alter the flexibility of the α-actinin dimer (Corgan et al., 2004). Thus, the three *Drosophila* α-actinin isoforms may well be differently regulated by phosphoinositides.

7.9. Ectopic Dpp signalling perturbs cytoskeletal remodelling (unpublished data)

Actn$^{\Delta 233}$;E4-GAL4/UAS-*dpp* egg chambers stained with anti-α-actinin frequently showed weaker staining in posterior cells, i.e. cells with ectopic Dpp signalling (Fig. 6I in III). The same was observed when UAS-*dpp* was overexpressed in the wild type background.

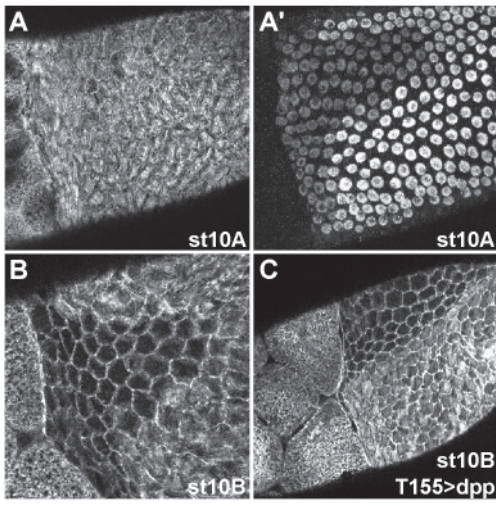

Figure 14. (A,B) Wild type. (A) All columnar follicle cells have basal actin fibres at stage 10A. (A') The dorsal midline cells are recognised by their lower level of Broad protein. (B) Dorsal midline cells have lost their basal actin fibres by stage 10B. (C) When Dpp is overexpressed, all dorsal cells (upper half) downregulate their basal actin fibres.

Therefore, α-actinin protein expression was examined in egg chambers with ectopic UAS-*dpp* expression throughout the follicular epithelium using the driver T155-GAL4. Eggs from T155-GAL4/UAS-*dpp* flies are fully elongated, lack dorsal appendages and have an operculum extending over more than half of the egg length on the dorsal side. During stage 10, the future operculum becomes visible as a downregulation of the transcription factor Broad (Dequier et al., 2001) and as a decrease in the density of the basal actin fibres. In wild type egg chambers, only dorsal midline cells downregulate their basal actin fibres (Fig. 14). It turned out that the seemingly reduced α-actinin staining was due to a substantially reduced posterior accumulation of α-actinin in the ventral follicle cells at stages 11 and 12 (Fig. 15A). The localisation of Ena was similarly affected (Fig. 15B). By contrast, the lateral accumulation of α-actinin at stage 13 appeared normal (not shown). Thus, ectopic Dpp signalling interferes with the cytoskeletal remodelling without affecting egg elongation. In light of these results it is easy to realise why in the wild type the anteriormost cells on the ventral side often showed reduced posterior accumulation of α-actinin and Ena: Only the first row of the centripetally migrating cells secrete Dpp, and the only cells transducing the Dpp signal in the absence of EGFR signalling are the anteriormost cells on the ventral side (Jékely and Rørth, 2003). Thus, some aspect of the cytoskeletal phenotype caused by ectopic Dpp signalling appears to be a normal feature of the centripetally migrating cells.

In order to test whether overexpression of α-actinin would counteract Dpp's negative effect on the formation of the posterior adhesion site, the localisation of Ena in T155-GAL4 B22/UAS-*dpp* follicle cells was examined. The result showed that ectopically expressed α-actinin could not overcome the negative effect exerted by ectopic Dpp signalling, in that a normal posterior patch still did not form. However, the increased amount of α-actinin did respond to some polarity cues within the cells and translocated more Ena to the posterior part of the cell (Fig. 15C). Thus, the A/P polarity within the cells is not lost by overexpression of Dpp even though the basal fibres do not reorganise properly. Furthermore, this result further supports the hypothesis that α-actinin plays a role in localising Ena at the posterior of the cell.

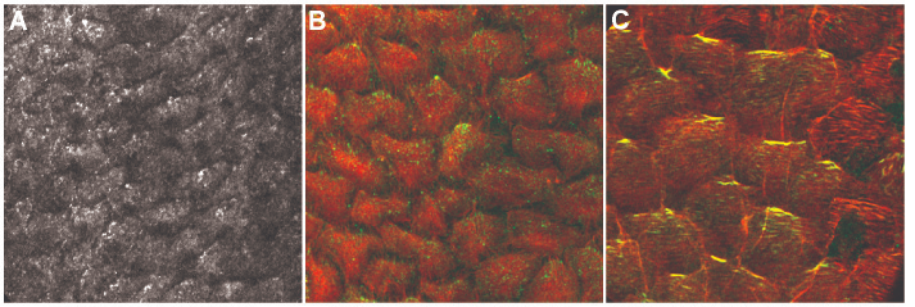

Figure 15. Ventral cells of stage 12 egg chambers overexpressing Dpp. The formation of a posterior patch containing α-actinin (A) and Ena (green in B) is blocked. (C) Co-expression of Dpp and adult muscle-specific α-actinin results in a larger amount of Ena (green) accumulating at the posterior of the cell, but the actin fibres (red) do not reorient.

7.10. A heat shock induces cytoskeletal alterations (unpublished data)

In some of the clonal experiments a nuclear Myc-tag was used as a marker. Expression of Myc was induced by a one hour heat shock at 37°C 90 minutes prior to dissection. In these experiments, the α-actinin pattern in stage 11-12 main body follicle cells repeatedly differed from the normal. Instead of the posterior patch, a single α-actinin-containing spike was seen. The heat shock alone was responsible for the induction of spikes, because wild type heat shocked flies showed the same pattern. The spikes contained F-actin and also accumulated Ena (Fig. 16). The lateral accumulation of α-actinin in stage 13 follicle cells was not affected, nor were any spikes observed in stage 10 egg chambers (not shown). This suggests that the induction of spikes is coupled to the remodelling of the cytoskeleton that occurs at stage 10B/11. Thus, the use of a heat shock-inducible clonal marker in studies of cytoskeletal dynamics in late stage follicle cells is clearly unsuitable. Previously demonstrated heat shock responses in the follicle cells are upregulation of Hsp27 (Marin and Tanguay, 1996) and activation of MAPK/ERK (Dammai and Hsu, 2003). MAPK/ERK was presumably activated also when EGFR signalling was ectopically activated throughout the follicular epithelium (Shilo, 2005), but this did not perturb the cytoskeletal remodelling. Thus, MAPK/ERK signalling alone is likely not the cause of the observed phenotype.

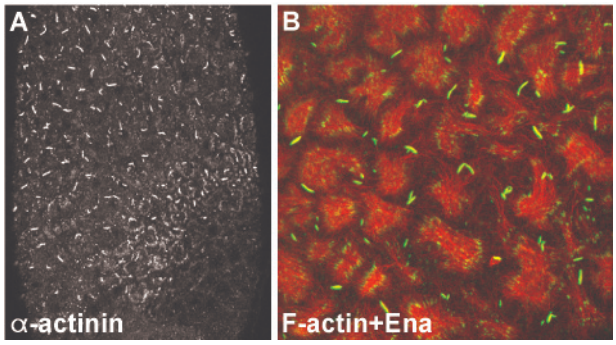

Figure 16. A heat shock results in the appearance of F-actin (red) spikes that contain α-actinin and Ena (green). (A) Stage 11 egg chamber stained with anti-α-actinin. (B) Close-up view of phalloidin- and anti-Ena-stained main body follicle cells.

CONCLUDING REMARKS

Drosophila has traditionally been used as a genetic model organism, in which new genes have been discovered via mutations that disrupt a specific developmental process. Malformed bristles, misshaped eggs and nonfunctional flight muscles have uncovered several actin regulators in *Drosophila*, and these organs therefore provide us with good model systems in which to examine the function and regulation of actin-binding proteins.

In this work, *Drosophila* Twinfilin was identified and shown to be functionally similar to yeast and mammalian twinfilins. The current model describing twinfilin's role in actin dynamics proposes that twinfilin localises ADP-actin monomers at sites of rapid actin assembly by interacting with capping protein. The characterisation of a strong hypomorphic *twinfilin* mutant revealed a strong bristle phenotype, which was due to excess actin polymerisation. This is consistent with the in vitro data showing that twinfilin sequesters monomers. Overexpression of Twinfilin did not cause a detectable phenotype, but could rescue the *twinfilin* mutant bristle phenotype. Thus, the bristle will be a good model system for further studies on Twinfilin function and regulation. By expressing various mutated Twinfilins and examining their ability to rescue the mutant phenotype, regions important for Twinfilin function are expected to be identified.

The second protein studied in this thesis project is α-actinin. Novel *Actn* mutant alleles were recovered that specifically disrupted the non-muscle α-actinin isoform. The $Actn^{\Delta 233}$ mutant was viable and fertile, and no phenotype that could be attributed to the lack of non-muscle α-actinin was observed. One reason for this may be that there is redundancy between actin crosslinking proteins. In addition, α-actinin may be important for normal cellular behaviour only under certain conditions not prevailing in the laboratory, as was observed for *Dictyostelium discoideum* α-actinin. Mammalian α-actinin interacts with and regulate the activity of several ion channels, including some expressed in the CNS. If this is true for *Drosophila* α-actinin as well, there might be some behavioural defect in the $Actn^{\Delta 233}$ mutant yet to be identified.

Comparison of the α-actinin expression patterns between wild type and $Actn^{\Delta 233}$ mutant animals revealed that the tissue-specific regulation of α-actinin expression is more complex than previously thought. Certain non-muscle cell populations, such as the main body follicle cells, express FC-α-actinin in addition to the non-muscle α-actinin isoform. Indeed, removal of all three α-actinin isoforms from the main body follicle cells resulted in a failure to remodel the basal cytoskeleton during egg elongation. This is the first phenotype to be identified in a *Drosophila* non-muscle tissue deficient in α-actinin. The experimental data lent support to the hypothesis that FC-α-actinin may include adult muscle-specific α-actinin, but ultimate proof will require the generation of isoform-specific antibodies. A special feature of the cells in which FC-α-actinin expression was observed is the formation of dense actin bundles. Thus, there may be a connection between FC-α-actinin expression and a stably organised actin cytoskeleton. Non-muscle

α-actinin could instead be involved in more dynamic events. The in vitro properties of the various *Drosophila* α-actinin isoforms are a completely unexplored area, although the position of the alternatively spliced exons indicates that the three isoforms may exhibit different flexibilities. The findings that combined EGFR and Dpp signalling represses FC-α-actinin and that co-expression of adult muscle-specific α-actinin and the activated EGF receptor induces distinct cytoskeletal alterations provide the first clues about the mechanisms that regulate α-actinin expression and function in *Drosophila*.

This work also describes a novel feature of the main body follicle cells. The finding that the basal actin fibres undergo an organised remodelling during egg elongation challenges the current view that the parallel actin fibres help to prevent axial expansion. Furthermore, several experimental conditions were identified (i.e. lack of α-actinin, overexpression of Dpp and heat shock) that altered the cytoskeletal behaviour of these cells without disrupting the epithelial organisation. Elucidation of the signal that induces the remodelling must await future studies. Integrin-based adhesion in terms of adhesion dynamics has been extensively studied in cell culture systems, whereas in *Drosophila*, the emphasis has been put on the analysis of mutants in which various developmental processes are perturbed (Brown et al., 2000). Several of the classical focal adhesion molecules have been identified in *Drosophila*, and it will be interesting to learn how they are localised in the main body follicle cells during egg elongation. Vinculin and FAK are both considered as key players in adhesion site dynamics (Carragher and Frame, 2004; Chandrasekar et al., 2005), yet neither of them is essential in *Drosophila* (Alatortsev et al., 1997; Grabbe et al., 2004). Would they be required for proper cytoskeletal remodelling during egg elongation? Based on the data presented in this work I propose that the follicular epithelium could provide us with an easily accessible and genetically tractable in vivo model system suitable for studies on mechanisms involved in adhesion site dynamics.

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